University of Alberta

A hypothesis-generating gene microarray analysis of human eosinophils stimulated by IFN-γ

By

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Abstract

Eosinophils are thought to play a critical role in host defense mechanisms against parasites and in allergic diseases. Recently, it has been postulated that eosinophils also play a role in viral infection and immunomodulation. IFN-γ is a Th1 cytokine that has been shown to stimulate a number of eosinophil functions including cytoxicity, priming, and mediator release. However, the effect of IFN-γ on eosinophil gene expression remains to be elucidated.

Using GeneChip microarray technologies, this study investigated the changes in the mRNA expression profile of eosinophils following IFN-γ (4 h) stimulation. Of the 100 genes found to be upregulated (> 3-fold), many were shown to play a role in immune/inflammatory response, signal transduction, apoptosis, and transport mechanism. In particular, a number of genes involved in innate immune response including TLR7 (9-fold), TLR8 (30-fold) and guanylate binding protein (GBP) (9-17-fold) were upregulated significantly. Testable hypotheses were generated as a result of this study.

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List of abbreviations

AAT	alpha-1-antitrypsin
APC	antigen-presenting cells
ASM	airway smooth muscle
EAR	early asthmatic response
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EMCV	encephalomyocarditis virus
EPO	eosinophil peroxidase
ESTs	expressed sequence tags
FBS	fetal bovine serum
GAF	gamma-activated factor
GAS	gamma-activated sequence
GBP	guanylate binding protein
GCOS	GeneChip Operating Software
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPRs	G protein-coupled receptors
IDO	indoleamine 2, 3-dioxygenase
IL-3	interleukin 3
IL-5	interleukin 5
IFN-γR1	IFN-γ receptor α chain
IFN-γR2	IFN-γ receptor β chain
IRF-1	regulatory factor-1

ISGs	interferon stimulating genes
ISRE	interferon-stimulated response element
JAK	Janus kinases
LAR	late-asthmatic response
LPS	lipopolysaccharide
MBP	major basic protein
PAMPs	pathogen-associated molecular pattern
PLSCR4	phospholipid scramblase 4
PMD	piecemeal degranulation
RANTES	regulated and activation normal T-cell expressed and secreted
RT-PCR	reverse transcriptase-polymerase chain reaction
RSV	respiratory syncytial virus
SERPING1	C1 inhibitor, serine proteinase inhibitor
SNAREs	SNAP receptors
STAT	signal transducers and activation of transcription
TGF	transforming growth factor
TLRs	Toll-like receptors
TNF	tumor necrosis factor family
VAMP	vesicle-associated membrane protein
VEGF	vescular endothelial growth factor
VSV	vesicular stomatitis virus

CHAPTER I

INTRODUCTION

1.1. The eosinophil

1.1.1. Eosinophilopoiesis and differentiation

Eosinophils were first recognized by Paul Ehrlich in 1879 as cells with numerous granules that stain red with the acidic dye, eosin [1]. They are end-differentiated granulocytes derived from CD34+ hematopoietic stem cells that are capable of extensive self-renewal in the bone marrow [2]. They pass through several morphologically distinct stages before their release as mature cells in the peripheral blood [2]. This process is closely coordinated by expression of cytokines and colony stimulating factors, their receptors and transcription factors [2]. Early eosinophil progenitor proliferation and differentiation involve a number of cytokines, some of which lack lineage specificity, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) [3]. However, terminal differentiation of the eosinophil lineage is dependent on interleukin-5 (IL-5) [3]. *In vitro*, treatment with anti-IL-5 blocks the eosinophil lineage commitment and differentiation of progenitors to mature eosinophils [4]. Anti-IL-5 antibody given to parasitized mice prevented the development of an eosinophilic response [5]. A similar observation is noted following administration of intravenous anti-IL-5 neutralizing antibodies in humans [6;7]. Conversely, blood eosinophilia is associated with increased levels of IL-5 [8].

After maturation in the bone marrow (approximately 8 days) eosinophils enter the bloodstream for a relatively short period of 13 to 18 h [9] and subsequently migrate to various tissue sites. The latter include the gastrointestinal tract, and skin where they reside for at least one week [10]. Eosinophils do not home naturally to the lungs in the absence of an allergic-type inflammatory response [10]. The number of eosinophils in the blood of healthy individuals is normally quite low, ranging between 1-3% of total leukocytes [10]. In atopic individuals, however, the numbers of peripheral blood eosinophils are often elevated [11].

1.1.2. Eosinophil morphology

Peripheral blood eosinophils are cells usually with bilobed nuclei, although three or more lobes are not uncommon [2]. Eosinophil cytoplasmic granules are stainable by eosin and are a main distinguishing feature of these cells. At least three types of cytoplasmic granules (secondary granules, small granules, and primary granules) have been characterized in eosinophils [9]. In particular, eosinophils are recognized by their unique large crystalloid granules, also known as secondary granules [2]. These granules contain at least 4 major cationic proteins as well as pro- and anti-inflammatory cytokines, chemokines and growth factors (Figure 1) [12]. Major basic protein (MBP), a cytotoxic protein known to damage a wide range of mammalian cells and metazoan parasites [13;14], is localized to the electron dense core of crystalloid granules, whereas the other three basic eosinophil proteins, eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN)

and eosinophil cationic protein (ECP), are stored in the matrix compartment of crystalloid granules [9].

1.2. Role of eosinophils in human disease and host defense

Since the identification of eosinophils by Paul Ehrlich in 1879, much has been learned about the biology of eosinophils including their critical role in innate host defense mechanisms and the association with most allergic diseases [9;12]. An elevated number of eosinophils is a feature of atopic dermatitis, allergic rhinitis, asthma and parasitic helminth infections [12;15-17]. Indeed, helminthic infections cause an increase in the number of circulating eosinophils and a massive tissue eosinophilic infiltration at mucosal sites where the parasite is localized [18;19]. It is thought that eosinophil recruitment to inflamed lesions is a critical component in host defense against such parasites, possibly because the cationic proteins (EDN, MBP, ECP, EPO) are cytotoxic against both adult and larval stages of helminths [20-22]. In contrast to the assumed beneficial role in host defense, eosinophils are thought to act as harmful effector cells in the pathogenesis of allergic diseases including allergic asthma [12]. Notwithstanding, current views are unclear about the precise role of this cell phenotype in immune and inflammatory responses [23].

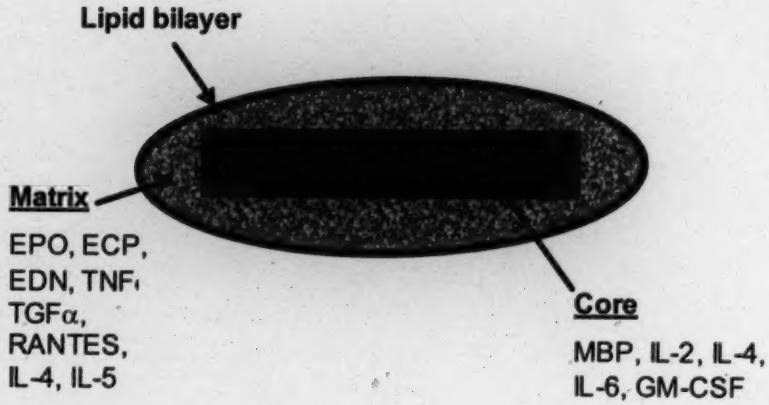


Figure 1. The specific (crystalloid) granule of eosinophils
(Modified from; a schematic design by the Moqbel laboratory)

1.2.1. The role of eosinophils in the pathogenesis of asthma

Asthma is a complex airway disease characterized by bronchial hyperreactivity, reversible airflow obstruction, airway inflammation, and airway remodeling (deposition of connective tissue proteins and development of fibrosis) [12;24]. The acute allergic reaction usually occurs within 3-5 minutes after re-exposure to an allergen in specific, and pre-sensitized subjects and is generally referred to as the early asthmatic response (EAR) [12]. EAR is followed, 2 to 6 hours later, by the late-asthmatic response (LAR), which involves infiltration of the airways by eosinophils, mononuclear cells, and lymphocytes as well as the elevation of Th2 cytokine and chemokine products (Figure 2) [12]. Many inflammatory cells are recruited and activated during airway inflammation in asthma, including eosinophils, which are found in large numbers and thought to be associated with increased severity of symptoms and asthma exacerbations [25;26]. A marked elevation in mucosal eosinophils and deposition of eosinophilic granule proteins in the bronchi were observed in patients dying of severe asthma [27;28]. Eosinophils are believed to cause long-term mucosal tissue damage when activated by releasing their granule contents.

In addition, eosinophils synthesize, store and release various pro-inflammatory cytokines, chemokines and potent cytotoxic cationic proteins following stimulation [9]. At the site of allergic inflammation, eosinophils release their granule content to extracellular spaces through a process known as exocytosis [29]. The release of mediators is thought to contribute to

the pathophysiology of asthma and associated tissue damage [30]. The capacity of these proteins to damage airways epithelium has been demonstrated both *in vitro* and *in vivo* [31;32].

One of the characteristic features of asthma is excessive airway mucus production due to goblet-cell hyperplasia and thickness of the airway wall, otherwise known as tissue remodeling [12]. Airway remodeling results from excessive repair processes following repeated airway injury, seen by an increase in airway smooth-muscle mass, the deposition of collagen and other matrix proteins and new blood-vessel formation [12;33]. Eosinophils were implicated airway remodeling, since they are a source of several fibrogenic and growth factors including transforming growth factor (TGF)-α, TGF-β and vescular endothelial growth factor (VEGF) [12]. Using eosinophil-lineage depletion in mice, a recent study demonstrated that eosinophils contribute substantially to airway remodeling by preventing collagen deposition and airway smooth muscle (ASM) mass [33]. Another study demonstrated that IL-5-deficient mice were protected from collagen deposition due to a reduction in TGF-β-positive eosinophils [34]. Human studies using antibodies against IL-5 also support a role for eosinophils in airway remodeling [35;36]. As indicated above, IL-5 is a key cytokine in eosinophil recruitment and activation at sites of inflammation [36]. One study demonstrated that mild asthmatic patients treated with IL-5-specific antibody for two months exhibited significant reduction in the expression of tenascin, lumican, and pro-collagen III in the bronchial mucosal reticular basement membrane [36].

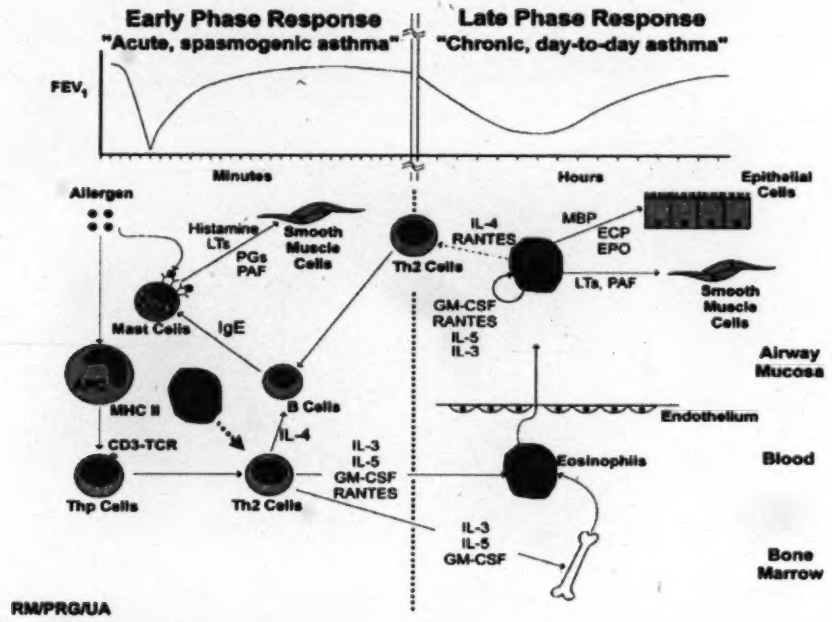


Figure 2. The current paradigm of the pathogenesis of both early and late phase asthmatic responses
(Modified from: *Moqbel R. 1999. Can. Respir. J. 6 (5) 453-457*)

Thus, eosinophils may play a possible role in the repair and remodeling processes.

1.2.2. The emerging role of eosinophils in immunoregulation

Eosinophils are classically considered as effector cells in pathogenesis of allergic disease and parasitic infection through the release of granular proteins and pro-inflammatory mediators [37]. However, there is emerging evidence that eosinophils may have immunoregulatory roles [37]. First, the release of certain mediators by eosinophils could potentially promote Th2 phenotype of inflammation at local sites [23]. Eosinophils have been known to synthesize, store and secrete various cytokines and chemokines including IL-4, IL-13 and regulated upon activation, normal T-cell expressed and secreted (RANTES) [23], mediators that have been strongly associated with the development of the asthmatic phenotype of inflammation [23]. Also, the release of TGF-β by eosinophils has been shown to contribute to airway remodeling [23].

Eosinophils may also have a direct influence on the function of lymphocytes [23]. In neonates, eosinophils home naturally to the gastroinstestinal tract, thymus, spleen, lymph nodes and mammary glands [38-40]. Whether the natural homing of eosinophils to the thymus is suggestive of a potential role in T-cell selection is still unknown [41]. However, such a notion is supported by the observations that eosinophils can act as antigen-presenting cells (APC) to activate T-cells [37;42]. One study reported that antigen-loaded eosinophils migrate to lymphoid tissues following

intratracheal administration into mice and present antigens to appropriate lymphocytes [42]. In addition, the expression of co-stimulatory molecules essential for interaction with lymphocytes including MHC II, CD80 and CD86 have been shown to be expressed by eosinophils [43].

Moreover, eosinophils may directly influence T-helper cell bias and function through a mechanism involving IFN-γ-inducible indoleamine 2, 3-dioxygenase (IDO), a rate-limiting enzyme in the oxidative catabolism of tryptophan (W) [44]. Our own laboratory has recently demonstrated that human peripheral blood eosinophils expressed the IDO enzyme constitutively. This enzyme has been previously identified as a critical factor in determining apoptosis of T-cells [45;46]. The findings indicated that eosinophil-derived IDO and its resultant catabolites of tryptophan, especially kynurenines, may contribute to suppression of Th1 cell proliferation, thus influencing the polarization of T-cells to Th2 phenotype in allergic subjects [44].

1.2.3. The emerging role of eosinophils in innate immunity

In addition to their proinflammatory roles in asthma and recently emerging immunomodulatory roles in acquired immunity, eosinophils may also play a role in innate immunity by mechanisms involving the activation of Toll-like receptors (TLRs). TLRs, homologues of Toll receptor in Drosophila, induce the expression of genes that control innate and adaptive immune responses by recognizing highly conserved microbial components known as pathogen-associated molecular patterns (PAMPs) [47]. Ten subtypes of human TLRs have been identified so far. These TLRs bind a remarkably

diverse array of bacterial, viral and fungal molecular patterns (Figure 3) [48]. TLRs recognize multiple PAMPs, including lipopolysaccharide (LPS) (detected by TLR4), and unmethylated CpG DNA of bacteria and viruses (detected by TLR9) [49]. TLRs 1, 2, 4, 5, and 6 are found on the cell surface and appear to specialize in the recognition of mainly bacterial products [49]. On the other hand, TLRs 3, 7, 8, 9 are localized to intracellular compartments and detect viral nucleic acids, including double-stranded DNA (TLR3), and single-stranded viral RNA (TLR7) [50].

Eosinophils are known to constitutively express mRNA for TLR1, TLR4, TLR7, TLR9 and TLR10 [51] although little information is known about their functions except for TLR4. However, increasing evidence suggests that eosinophils lack TLR4 protein expression on the cell surface and they are nonresponsive to LPS stimulation [52;53]. On the contrary, eosinophils respond to stimulation with R848 (an activator of TLR7), resulting in their activation and prolonged survival [51]. R848 is a synthetic ligand for both TLR7 and TLR8 [51]. This finding suggests that eosinophils may be involved in the exacerbation of allergic inflammation during viral infection. Many studies have shown that infections with respiratory viruses are potent triggers of acute exacerbations of asthma [54]. In fact, up to 80% of exacerbations of asthma in children and about 50% in adults are associated with respiratory viral infections [54]. Regardless, the pathogenetic events underlying the association between respiratory infections and asthma exacerbations are not completely understood.

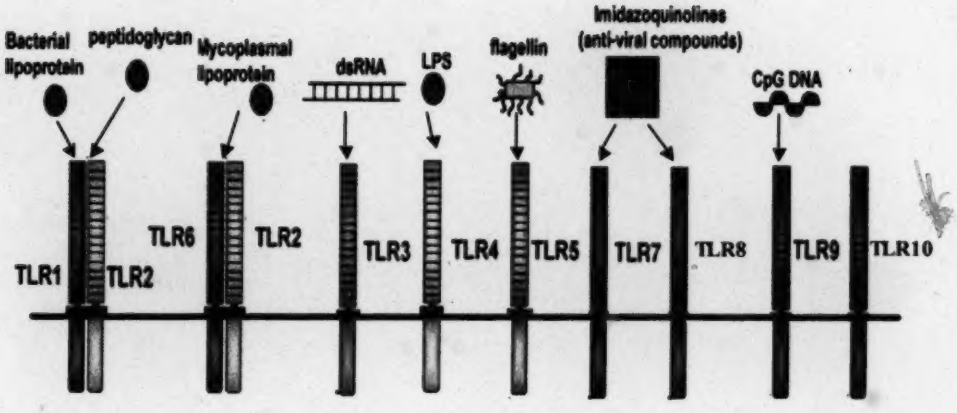


Figure 3. TLRs recognize molecular pattern associated with bacterial pathogens. triacylated lipoprotein for TLR1; peptidoglycan for TLR2; double-stranded RNA for TLR3; lipopolysaccharide (LPS) for TLR4; flagellin for TLR5; diacylated lipoprotein for TLR6; imidazoquinoline and its derivative R-848, for TLR7 and TLR8; bacterial unmethylated CpG DNA for TLR9; and unknown ligand for TLR10.
(Modified from: *Akira et al., Molecular immunology 2004. 861-868*)

1.3. Eosinophils and cytokines

Although eosinophils are known as non-proliferating, end-differentiated cells, they possess the capacity to synthesize a number of proteins. Human eosinophils, as indicated by many studies, can produce and release cytokines (IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF, TNFα, IFN-γ) and chemokines (IL-8, MIP-1α, RANTES, eotaxin) as well as growth factors (TGF α/β, PDGF, NGF) among others [55]. As such, eosinophils may release preformed regulatory proteins and growth factors locally and rapidly during inflammation. The release of these cytokines further supports the notion that eosinophils can act as pro-inflammatory cells.

Although many inflammatory cells such as mast cells, and macrophages secrete cytokines that influence eosinophil differentiation, survival, and function, T cells, central cells in the adaptive immune response, are thought to have a central role in the activation of eosinophils by releasing cytokines [56]. Th1 cells, with a cytokine release profile of IFN-γ, IL-2, IL-12, IL-18, and TNF, are important in activating macrophages and inducing cell-mediated immunity [57]. On the other hand, Th2 cells, with a cytokine profile of IL-4, IL-5, IL-6 IL-10, IL-13 together with IL-3 and GM-CSF (shared by Th1- and Th2-type T cells), are important in humoral immunity and allergic inflammation [57]. Cytokines produced by Th2-type cells are classically thought to activate eosinophils. This view has evolved during the years as more studies revealed that eosinophils are influenced by Th1 type cytokine such as IFN-γ [58;59]. IFN-γ is produced mainly by Th1 cells and is usually

associated with bacterial and viral infections. IFN-γ has been known to stimulate eosinophils *in vitro*, as demonstrated by studies on the expression of FcγRIII (CD16) and CD69 [59]. Furthermore, IFN-γ induces the release of various cytokines from eosinophils, including GM-CSF, IL-6, and RANTES [60;61], as discussed later in section 1.5.1.

1.4. IFN-γ biology and signaling

1.4.1. IFN-γ and its receptor

IFN-γ was first recognized nearly 40 years ago as a protein capable of protecting cells from viral infection [62]. IFN-γ is synthesized mainly by activated natural killer (NK) cells, activated T helper cells of the Th1 subset, and activated $CD8^+$ cytotoxic cells in response to a number of immune and inflammatory stimuli [63]. IFN-γ is known to play an important role in host defense, inflammation and autoimmunity [64].

Human IFN-γ is a non-covalent 34-kDa homodimer that consists of two identical 17-kDa polypeptide chains [65]. X-ray crystallographic structure of human IFN-γ has revealed the dimeric nature of the mature protein and shows that two polypeptides self-associate in an anti-parallel fashion thus exhibiting a two-fold axis of symmetry [66]. This suggests that a single IFN-γ homodimer molecule can bind two IFN-γ receptor molecules [62]. Experimental data has indicated that full biological activity is exerted only by the homodimeric form of the protein [64].

Each IFN-γ receptor consists of two species-matched subunits, IFN-

γR1 (otherwise known as IFN-γ α) chain, and IFN-γR2 (IFN-γ β) chain (Figure 4) [67]. Immunochemical and radioligand binding experiments have suggested that IFN-γ α chain, a 90-kDa glycoprotein, is the major ligand-binding subunit [68]. IFN-γ β chain, a 60-67-kDa glycoprotein is presumed to increase the affinity of IFN-γ α chain for its ligand [68]. This is achieved by enhancing the stability of the complex, however, it does not play an important role in direct ligand binding [68]. Nevertheless, the β chain is required for the transduction of the IFN-γ signal [62]. Two independent groups in 1995 demonstrated that the lack of cellular expression of the β chain results in unresponsiveness to IFN-γ [62].

1.4.2. IFN-γ signaling : classical JAK-STAT pathway

In the process of defining the mechanisms of IFN-γ receptor signaling, two distinct protein classes were identified as key molecules mediating IFN-γ-dependent cellular responses [62]. These transducers are members of the Janus kinases (JAK), a family of nonreceptor tyrosine kinases, as well as a novel family of cytoplasmic transcription factors termed signal transducers and activation of transcription (STAT) [62]. These latter proteins play a dual role, first as a signal transducer by acting as substrates for the JAKs, and after phosphorylation, dimerization, and nuclear translocation, as transcriptional activators resulting in gene transcription [69]. Thus, IFN-γ mediates cellular responses through its heterodimeric cell-surface receptor (IFN-γR), which activates downstream signal transduction cascades, ultimately leading to the regulation of IFN-γ-induced gene expression [68].

However, unlike other major growth factor receptors, the IFN-γ receptor α chain and β chain (members of the class 2 cytokine receptor family) lack intrinsic kinase activity [62]. Thus, IFN-γ mediates signaling through other protein kinases, in this case, JAK1 and JAK2 [68].

The JAK family of proteins consists of four members, JAK1, JAK2, JAK3 and TYK2 [62]. Among these, only JAK1 and JAK2 are required for IFN-γ signaling (Figure 4) [70].

As mentioned above, among the best-characterized JAK substrates are STAT proteins [71]. Originally described by Darnell and co-workers, STAT proteins play a critical role in signal transduction pathways associated with IFN-γ [62]. Among the seven members activated by distinct cytokine receptors, only STAT1 is tyrosine phosphorylated in response to IFN-γ (Figure 4) [72].

In unstimulated cells, the α and β subunits of the IFNγ receptor do not pre-associate with each other, but constitutively associate with inactive forms of JAK1 and JAK2, respectively [68]. *In vitro*, IFN-γ, a homodimeric ligand, promotes rapid oligomerization of the two IFN-γ receptor subunits and brings into close juxtaposition the intracellular domains of these proteins with the inactive JAK enzymes they carry. Once clustered, JAK proteins are reciprocally activated through sequential cross-phosphorylation events [73]. Activated JAK2 phosphorylates the functionally critical Y_{440} residue near the C-terminus of the IFN-γ α subunit, thereby forming a paired set of STAT1 docking sites on the ligated receptor [62]. This phosphorylated docking site,

embedded within the recognition sequence ($_{440}$YDKPH$_{444}$), is bound by STAT1 through its SH2 domain [68]. The docking of STAT1 molecules to the IFN-γ α chain allows phosphorylation on tyrosine residue Y_{701} by activated JAKs [72]. The phosphorylated STAT1 proteins homodimerize via reciprocal SH2-phosphotyrosine interactions, forming a protein complex, gamma-activated factor (GAF) [68]. This is followed by the translocation of the homodimer complex of STAT1 to the nucleus, where binding occurs to a nine-nucleotide consensus sequence, TTNCNNNAA, known as gamma-activated sequence (GAS) [68].

1.5. IFN-γ signaling in human eosinophils

1.5.1. Early and late events

IFN-γ has been reported to regulate a number of eosinophil functions [58-61;74]. Conventionally, IFN-γ is thought to exert influence over protracted period of incubation (12-24 h) since regulation by transcription factors results in new protein synthesis [58]. Previous studies showed that IFN-γ could influence human eosinophil cytotoxicity, and receptor expression following long-term (minimum 4 h to reaching maximum at 24 h) stimulation [58;59]. In addition, IFN-γ was found to upregulate RANTES mRNA and protein expression in eosinophils after 16 hours of stimulation [56]. Another study demonstrated that RANTES mRNA expression was depleted 1-4 h after IFN-γ stimulation and then was replenished after 24 h [75]. Recent studies, however, have suggested rapid effects (within 15 minutes) of IFN-γ on

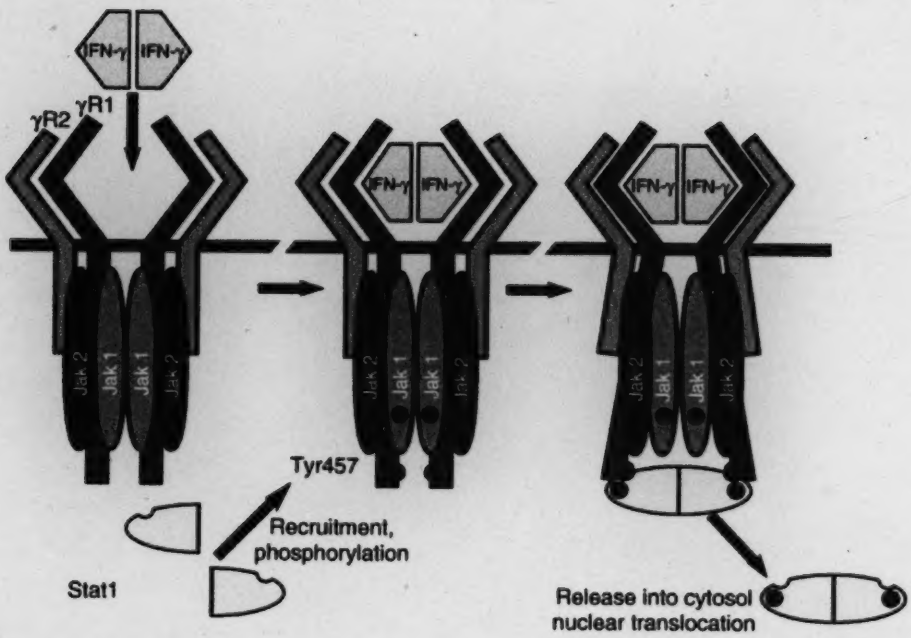


Figure 4. The IFN-γ receptor complex. The IFN-γ receptor complex consists of two different chains, IFN-γR1 and IFN-γR2. Upon ligand binding, the Jak kinases cross-phosphorylate each other and the activated Jak kinases then tyrosine phosphorylates each IFN-γR1 chain, thereby recruiting and activating STAT1. Once phosphorylated, STAT1 is translocated to the nucleus to activate IFN-γ regulated genes
(Modifed from: *Pestka et al., Immunological Reviews 2004. 8-32*)

cytokine expression, and mediator release in eosinophils [60]. Our own lab showed that IFN-γ rapidly (10 min) elevated IL-6 immunoreactivity in human peripheral blood eosinophils [61]. Moreover, IFN-γ was implicated to play a role in rapid (15 min) mobilization and release of eosinophil-derived RANTES from human eosinophils [60;76]. In a study from our own group, genistein, a broadly specific tyrosine kinase inhibitor, at 10^{-6}M, fully inhibited activation of RANTES mobilization and release from eosinophils by IFN-γ [60]. This is consistant with IFN-γ acting on these cells via its IFN-γ receptor, which in turn activates the JAK-STAT pathway [60]. Based on these findings, it is conceivable that IFN-γ or IFN-α/β released from virus-infected immune cells in the airways of asthmatics may activate eosinophils during viral exacerbation of asthmatic attacks. Thus, it may not be surprising that IFN-γ, a Th1-type cytokine, may have a role in regulation of eosinophils, generally regarded as a Th2-type effector cells.

1.5.2. IFN-γ signaling in eosinophils

Human eosinophils express functional IFN-γ receptors and appear to rapidly form a complex of the two receptor subunits on the cell membrane in response to IFN-γ [77;78]. Ishihara *et al.* demonstrated that eosinophils, obtained from both asthmatic and healthy individuals, expressed both IFN-γR α and β subunits by flow cytometric analysis [77;78]. Coimmunoprecipitation studies revealed rapid (1 min) formation of complex between IFN-γ α and β chains following IFN-γ stimulation of cells [78]. Further, Ochiai and colleagues reported rapid tyrosine phosphorylation of JAK1, JAK2 and STAT1α in

human peripheral eosinophils after receptor dimerization [79]. The latter study showed that within 3-10 minutes following exposure to IFN-γ, all known components of the signaling cascade were maximally activated [79]. Tyrosine kinase activity has been implicated in eosinophil activation and mediator release [60;79]. Little is known regarding the specific signaling mechanisms that are associated with IFN-γ stimulation in eosinophils.

1.6. Gene arrays in the study of cytokine effects on cells

1.6.1. DNA Microarrays

With the increasing availability of human cDNA sequence information and the advent of microarray technologies, scientists are provided with the unique opportunity to ask broad questions concerning the contribution of gene expression to phenotypic changes in cells [80]. Microarrays of oligonucleotides or cDNAs can be used to measure the expression levels of thousands of genes simultaneously in a single experiment and have several areas of application including gene expression profiling, polymorphism analysis and sequencing [80]. For example, microarrays can be used in gene expression profiling, in which the expression levels of genes are measured in different physiological conditions both in cultured cells and tissues, to search for regulatory expression patterns [80]. This enables scientists to compare gene expression in cells or tissues taken from patients and controls, or from patients before and after clinical intervention [81]. Understanding patterns of expressed genes may improve our knowledge of highly complex networks of

biological processes both in health and disease. Further, this could lead potentially to the identification of new disease mechanisms, and eventual therapeutic targets.

1.6.2. Microarray technology

Although many different microarray technologies are available, current literature is dominated by GeneChip Oligonucleotide arrays (Affymetrix, Santa Clara, CA) and spotted cDNA arrays, with use of short oligomers (25 bases), and longer fragments of cDNA, respectively [82]. The basic principle is the same for most microarrays with a probe immobilized on a surface and a target made from RNA isolated from the sample of interest [83]. Basically, the technique relies on the hybridization of the fluorescently labeled complementary DNA probes made from total mRNA to an array of DNA representing human genes [80]. Following the hybridization, the slide is laser-scanned, which causes excitation of fluorescently labeled cDNAs probes [80]. The fluorescence is measured and data are analyzed by appropriate software [83].

1.6.3. Microarrays in the study of IFN-γ stimulation in eosinophils

Few microarray studies have been done with respect to eosinophils to date. Among these studies, a major interest has been on the gene expression levels in eosinophils stimulated with IL-5, a major effector cytokine influencing the development, maturation and recruitment of eosinophils [84;85]. One microarray study identified a number of genes in IL-5-treated eosinophils that could potentially regulate eosinophil survival and

apoptosis [84]. Conversely, there is a paucity of data on the nature of genes regulated by IFN-γ in eosinophils. Until recently, little attention has been given to IFN-γ, a Th1 type cytokine, in the asthma research particularly in terms of linking IFN-γ to eosinophils due to Th2-biased characteristics of the immune response in asthma. However, IFN-γ has been implicated in a number of eosinophil functions [59;74]. Also, studies suggest that both Th1 and Th2 cytokines may be involved in allergen-induced airway inflammation, particularly in virally exacerbated asthma [86-88].

Given the importance of eosinophils in allergy and their responsiveness to IFN-γ, there is a vital need to investigate the global gene expression regulated by IFN-γ in human eosinophils. Such an approach would potentially lead to the generation of testable hypotheses in gene function of the cell. Also, it may lead to the expansion of our knowledge on the role of IFN-γ on eosinophils and the clinical and immunological consequences of such interaction both in terms of perpetuation of the Th2 profile but also in immunoregulation of allergic inflammation and asthma.

1.7. Rationale for the study

IFN-γ is a Th1 cytokine that has been primarily known to play a role in host defense against viral infections. In recent years, this cytokine has been implicated in the regulation of a number of eosinophil functions. In particular, reports suggested that IFN-γ has a short-term effect on human eosinophils [44;60]. IFN-γ induced short-term (15 min) effect on the release of eosinophil-

derived RANTES [60]. In addition, in our lab, we examined IDO mRNA expression following a 4 h (intermediate) incubation with IFN-γ [44].

The effect of IFN-γ on the eosinophil gene expression remains to be elucidated. Targeting certain genes regulating IFN-γ-induced modulation of eosinophil function will yield data that may be important in the understanding of eosinophil function and, eventually, the development of novel therapeutic strategies to modify eosinophil-associated pathologic changes in diseases such as asthma. Because of this, current study has focused on obtaining data on the interaction between eosinophils and IFN-γ as expressed in gene profiles.

1.8. Study objective

This is a hypothesis-generating study in which IFN-γ-inducible gene expression will be determined in human eosinophils from allergic subjects. The study will use a GeneChip (Affymetrix) microarray to show the effect of exposure of eosinophils to IFN-γ for 4 h.

CHAPTER II

Materials and Methods

2.1. Preparation of eosinophils

Eosinophils were purified from individuals with a range of peripheral blood eosinophilia, usually with a history of atopy, as previously described [60]. Briefly, a sample of 100 ml of peripheral blood was sedimented in 6% dextran for 30 min at ambient temperature. The leukocyte-rich supernatant was collected and layered onto Ficoll-paque (Amersham Biosciences, PQ, Canada) (50 ml blood/15 ml Ficoll gradient) and centrifuged for 20 min at 1200 rpm (~350g) at room temperature. After discarding the layer containing monocytes, the granulocyte pellet was resuspended in 1.5 ml of distilled water to lyse contaminating erythrocytes before adding 20 ml of RPMI-1640 containing 5mM EGTA (Buffer A). Cells were washed twice in Buffer B (Buffer A + 2% v/v FBS). The centrifuged pellets were resuspended in 2ml of Buffer B and incubated for 30 min at 4°C with a cocktail of CD16 (12 μl beads/ 5 x 10^7 cells), CD3 and CD14 immunomagnetic beads (10μl each) (Miltenyi Biotec, CA, USA) to remove neutrophils, lymphocytes and monocytes, respectively. Highly purified eosinophils (>99%) were obtained by negative immunomagnetic selection using AutoMACS (Miltenyi Biotec Inc. Auburn, CA).

Eosinophils may be activated by the isolation procedure. Therefore, purified eosinophils were kept in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) for 60 min at 37°C following isolation to bring

them back to optimal conditions. To determine eosinophil purity (>98%) as well as viability (>99%), Kimura and Trypan Blue stains were used, respectively, immediately following isolation and again following 1 h incubation.

2.2. *In vitro* Eosinophil stimulation with IFN-γ

IFN-γ was purchased from R&D systems (Minneapolis, MN). For polymerase chain reaction (PCR), reverse transcriptase-polymerase chain reaction (RT-PCR), and Real-time PCR analysis, approximately 3-5 x 10^6 purified eosinophils were treated with 200U/ml (20ng/ml) IFN-γ or medium alone for 4 h at 37°C.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from eosinophils (3 x 10^6 cells minimum/extraction) treated in the presence or absence of IFN-γ using the Qiagen QIAShredder and Qiagen RNeasy Mini kit (QIAGEN Inc, Mississauga, ON,). Extracted RNA was resuspended in RNase-free water (10μl/sample) and stored at –80°C until use.

RT-PCR was performed as follows: 100ng of total RNA was added to 1μl of oligo-$(dT)_{12\text{-}18}$ (0.5μg/μl) (Invitrogen) and 1μl mixed dNTPs (10mM) and made up to 12μl with RNase-free water. The mixture was heated for 5min at 65°C, followed by quick chilling on ice. The RT master mix consisting of 4μl of 5X First-strand buffer (Invitrogen), 2μl 0.1M DTT, and 1μl of M-MLVRT

(200U/μl) per reaction was added to the mixture and incubated for 50min at 37°C. Reactions were terminated at 70°C for 15min. The generated cDNA was either used immediately for PCR reactions or stored at –20°C for subsequent use.

2.4. Polymerase chain reaction (PCR)

To analyze indoleamine 2,3-dioxygenase (IDO) transcript expression, RT-PCR was carried out using IDO-specific primers. Two sets of primers were designed to span four intron regions and amplify a 230-bp of IDO cDNA (primer set 2), and three intron regions and amplify a 201-bp fragment of IDO cDNA (primer set 1), respectively. Control primers were directed at 18S ribosomal RNA. Primer sequences were as follows:

Primer set 1:

IDO: forward nt- 5'-CTGTGTCTTGGCAAACTGGA-3'

reverse nt- 5'-AGTGTCCCGTTCTTGCATTT-3'

18S rRNA: forward nt- 5'-GTAACCCGTTGAACCCCATT-3'

reverse nt- 5'-CCATCCAATGGGTAGTAGC-3'.

Primer set 2:

IDO: forward nt- 5'-AGAAGTGGGCTTTGCTCTGC-3'

reverse nt- 5'-TGGCAAGACCTTACGGACATCTC-3'

18S rRNA: forward nt- 5'-GTAACCCGTTGAACCCCATT-3'

reverse nt- 5'-CCATCCAATGGGTAGTAGC-3'.

Specificity and template alignment of primers was confirmed using the BLAST (NCBI) program available through the internet.

Aliquots (2μl) of cDNA were used in a 25μl PCR reaction containing PCR master mix. Reactions were carried out in a PTC 100 Thermal Controller (M-J Research) according to a 7 step program: (1) 10min at 94°C, (2) 20sec at 94°C, (3) 25sec at 55°C, (4) 25sec at 72°C, (5) repeat Steps 2-4 35 cycles, (6) 10min at 72°C, (7) 4°C. Amplified products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and scanned under ultraviolet light using the Alphalmager 2200 (Alpha Innotech. San Leandro, CA).

2.5. Real-time PCR

Amplification was performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). PCR fragments were separated on 2% agarose gel and visualized using ethidium bromide staining. PCR products from IDO and 18S ribosomal RNA (18S rRNA) fragements were gel purified using the Qiaquick gel extraction kit (Qiagen) and the concentration was determined colorimetrically to calculate the DNA copy number of the purified product. For quantitative PCR, amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Following activation of the *Taq* polymerase enzyme at 95°C for 15 min, the reactions were cycled 35 times at 95°C for 15s and 60°C for 1 min. Fluorescence was detected at 510nm following the extension step using the Rotor-Gene real-

time PCR detection system (Corbett Research). A standard curve was generated from the amplification of serial log dilution of the stock IDO and 18S rRNA PCR products and used to determine the efficiency of PCR amplification. Test samples were then amplified along with standards to quantify RNA. Following normalization with 18S RNA, the DNA copy number was calculated for each treatment. The amount of starting RNA was expressed as the mean transcript number following normalization with the copy number of the corresponding 18S RNA transcripts.

2.6. Microarrays

2.6.1. RNA extraction

Cells (8-10 x 10^6) were incubated according to the following treatment conditions: unstimulated cells (control), cells stimulated with IFN-γ at 37°C for 30min (test-1), unstimulated at 37°C for 4 h (control), stimulated with IFN-γ at 37°C for 4 h (test-2).

A cDNA library was constructed from purified human peripheral blood eosinophil by pooling mRNA from 15 to 20 donors (~1.0 μg of RNA in total). Pooling of total RNA samples was performed to compensate for the limited RNA sample quantities.

Following treatment, cells were pelleted (1200 rpm for 5min at room temperature) and total RNA was extracted as described above (Section 2.3) with the following modification to the manufacturer's protocol. Briefly, the cells (8-10 x 10^6) were lysed with 600μl of Buffer RLT (Qiagen RNeasy mini-

kit, Qiagen, Missasaga, ON.) and homogenized with Qiagen QIAshredder. An additional step where the samples were digested with DNase I (Qiagen) was incorporated to remove contamination from genomic DNA [89]. Extracted RNA samples were stored at –80°C until pooled.

2.6.2. RNA quality and quantity

To minimize RNA degradation and low yield, the extraction procedure was carried out as rapidly as possible after stimulation. Once extracted, RNA samples were frozen and thawed only once. Due to the limited amount of eosinophil RNA, it was not possible to run a denaturing agarose gel electrophoresis to examine the quality of RNA. The RNA 6000 LabChip kit and the Agilent 2100 Bioanalyzer allow effective measurement of both the quantity and the purity of RNA samples with minimal wastage (1μl) of sample. Therefore, the quantity and quality of RNA samples were effectively measured using the RNA 6000 LabChip Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The ratio of the peak areas of the ribosomal bands, 18S/28S, from the electropherogram, was automatically calculated using the Bioanalyzer, which provides information on the quality of RNA samples (Figure 5) [90]. The 18S/28S ratio of the RNA samples obtained in this study ranged between 1.0 – 1.3, thus meeting the criteria set by Institute of Biomolecular Design (IBD) at the University of Alberta.

All samples, except for unstimulated (0 min control) condition, generated sufficient amount of RNA (1μg – 1.7μg) of high quality to carry out the microarray analysis. After careful consideration, only two samples, 4 h

with/without IFN-γ, were put through the Affymetrix array process.

2.6.3. Sample and array processing

Human genome-wide gene expression was examined using the Human Genome U133 Plus 2.0 Array (GeneChip; Affymetrix, Santa Clara, CA.), which contains the oligonucleotide probe set for 54,000 full-length genes. Experiments were performed in accordance with the manufacturer's protocol (Expression Analysis Technical Manual, Section 2: Eukaryotic Sample and Array Processing, Affymetrix) (Figure 6). Total RNA isolated from eosinophils was precipitated with ethanol. Subsequently, double-stranded cDNA was synthesized by using One-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA.) containing T7-Oligo(dT)Primer, SuperScript II, *E. coli* DNA ligase, *E. coli* DNA Polymerase I, RNase H, T4 DNA Polymerase. Poly-A controls were added as exogenous positive controls to monitor the entire eukaryotic target labeling process. Following cleanup of double-stranded cDNA, GeneChip IVT Labeling Kit was used to synthesize biotin-labeled cRNA. At this stage, quantification of the cRNA was done using spectrophotometric analysis. Absorbance at 260nm and 280nm was checked to determine sample concentration and purity; A260/A280 ratio of 2.0 indicated that the cRNA samples were pure. The cRNA (15μg/sample) was fragmented by adding 5X fragmentation buffer that has been optimized to break down full-length cRNA into fragments (35 to 200 base) and incubating at 94°C for 35 min. Both cRNA and fragmented cRNA were assessed using an Agilent Bioanalyzer LabChip. Following fragmentation, the biotinylated

fragmented cRNA was hybridized with a U133 Plus 2.0 Array for 16 hours at

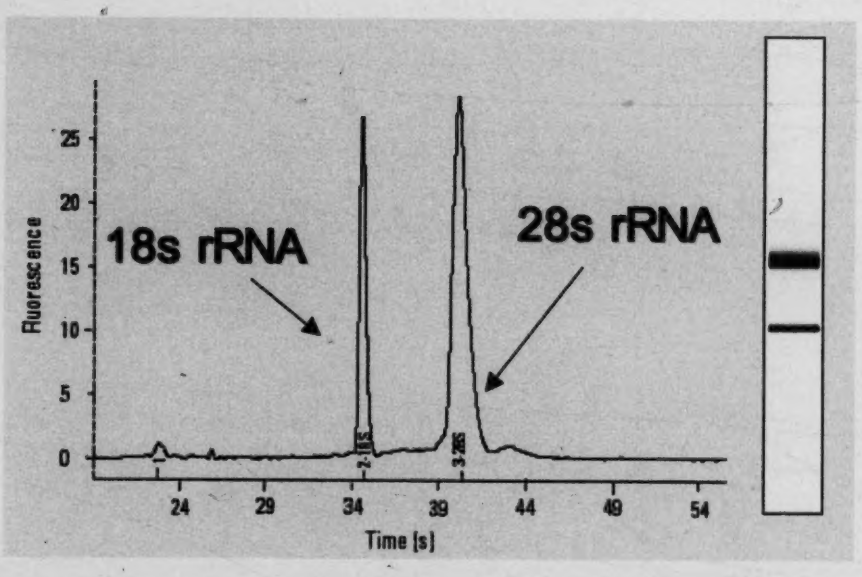


Figure 5. Electropherogram and gel-like image showing a high quality, eukaryotic, total RNA sample. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed
(Modified from: *an application note by Kuschel M., Agilent Technologies* [90])

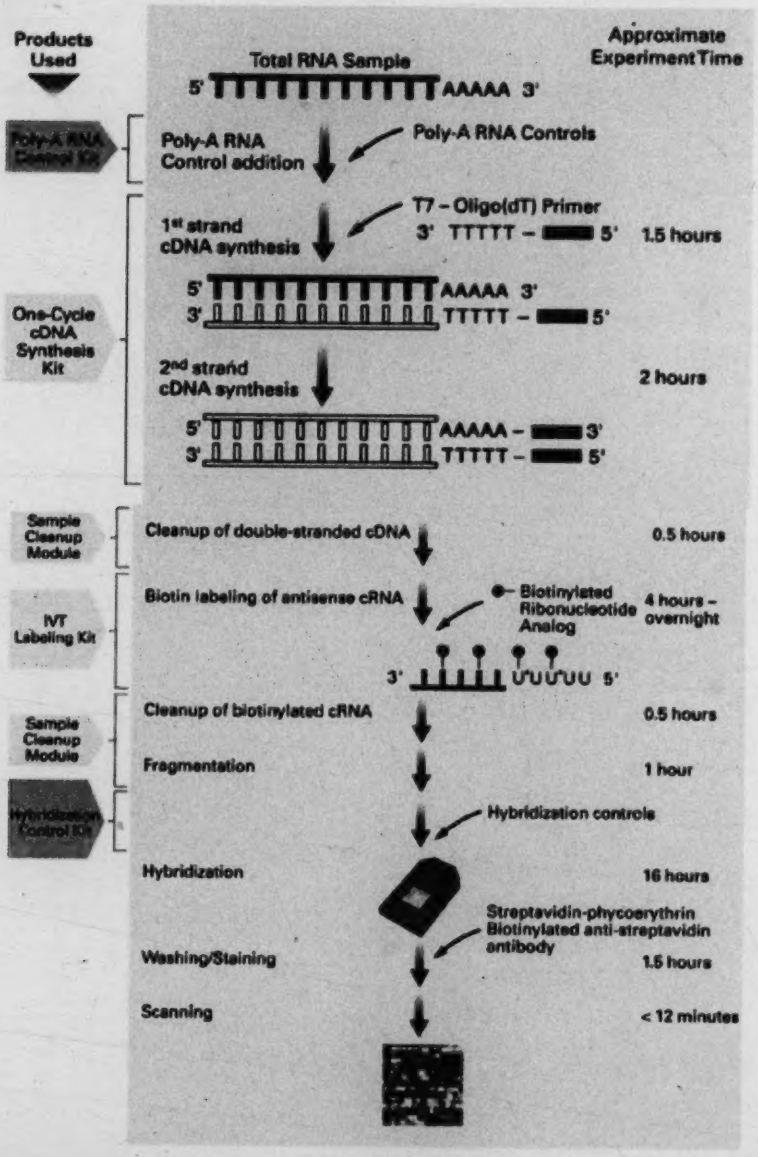


Figure 6. GeneChip eukaryotic labeling assay for expression analysis (Modified from: *Expression Analysis Technical Manual, Section 2: Eukaryotic Sample and Array Processing, Affymetrix*)

45°C. In order to monitor hybridization and staining, B2 Oligonucleotide control and 20X eukaryotic hybridization controls were added. After washing using the Fluidics Station 450, the hybridized biotinlyated fragmented cRNA was stained with streptavidin-phycoerythrin (1mg/ml) and then scanned with the Affymetrix GeneChip Scanner 3000.

2.6.4. Genechip expression analysis

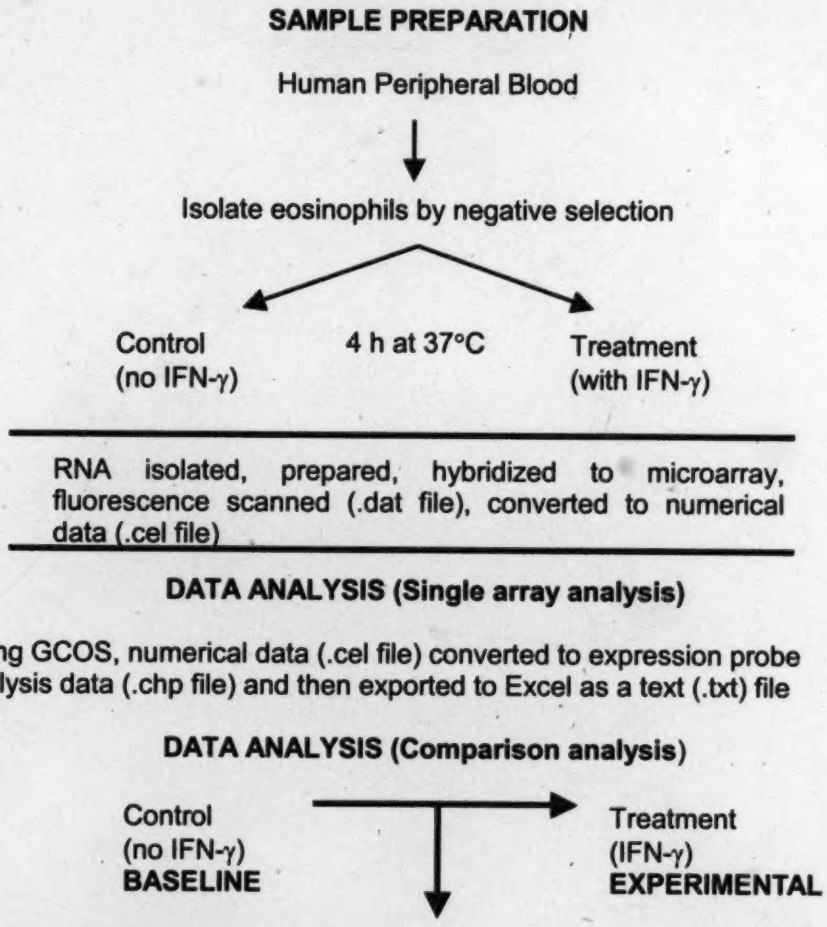
Affymetrix GeneChip is a silicon chip with a total of 11 oligonucleotide probe pairs for each gene/Genbank entry (Affymetrix GeneChip Expression analysis). Each probe pair consists of perfectly matched and single nucleotide-mismatched primers consisting of 25-base oligonucleotides. The fluorescence intensity of each probe was quantified using a computer program, Genechip Operating Software (GCOS) (Affymetrix, CA). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained by a probe set (11 to 15 pairs). The Affymetrix GCOS program recorded fluorescent signals as image data (.dat) files and converted them to numerical cell intensity (.cel) files. This was then converted to expression probe analysis data (.chp) file and exported to Excel as a text file. All the subsequent analysis were performed using the Excel file containing approximiately 54,000 genes. Initially, first-order data analysis called single array analysis is performed for each treatment (Figure 7). Based on the expression report (.rpt file) generated, it was found that normalization was not necessary. Normalization corrects for variations between two arrays, and in this case, the two arrays

were very close to each other, eliminating the needs for normalization. Following the single-array analysis, the comparison analysis between the two samples was performed, designating 4 h control as a baseline and 4 h with IFN-γ as experimental. Three metrics, namely, detection, change and signal log ratio were given for the data interpretation. Detection indicates presence or absence for a particular transcript. Change is a measure of increase or decrease signal for the transcript. Signal log ratio is the quantitative measure of the relative change in transcript abundance. From the signal log ratio, fold change was calculated following the formula below.

$$\text{Fold change} = \begin{cases} 2^{\text{signal log ratio}} & \text{Signal log ratio} \geq 0 \\ (-1) \times 2^{-(\text{Signal log ratio})} & \text{Signal log ratio} < 0 \end{cases}$$

Based on the fold change calculation, the 54,000 genes identified were sorted for robust changes (> 3-fold) indicating significant changes in gene expression. This eliminated the majority of genes leaving 697 upregulated genes and 775 downregulated genes to be analyzed. Once the comparison analysis was completed, GeneSpring software (Silicon Genetics, Redwood City, CA) (courtesy of Dr. Phil Halloran) as well as NetAffx (www.affymetrix.com) were used to determine known biological functions of each gene manually. GeneSpring links to bi-monthly updated, annotated genomic databases with all known human genes and expressed sequence tags (ESTs) to aid in discovery and reporting of newly identified genes. At the

end of the analysis, only 244 genes (upregulated) and 250 genes (downregulated) were identified with known gene functions.


SAMPLE PREPARATION
Human Peripheral Blood
Isolate eosinophils by negative selection
Control
(no IFN-γ)
4 h at 37°C
Treatment
(with IFN-γ)
RNA isolated, prepared, hybridized to microarray, fluorescence scanned (.dat file), converted to numerical data (.cel file)
DATA ANALYSIS (Single array analysis)
Using GCOS, numerical data (.cel file) converted to expression probe analysis data (.chp file) and then exported to Excel as a text (.txt) file
DATA ANALYSIS (Comparison analysis)
Control
(no IFN-γ)
BASELINE
Treatment
(IFN-γ)
EXPERIMENTAL

Search for the known biological functions of the genes using tools such as GeneSpring and NetAffx.

1. Fold change (3 fold and up)
   A. Upregulated genes (Appendix A Table A.1)
   B. Downregulated genes (Appendix A Table A.2)
2. Significantly changed genes (Top 100 fold change) under different catagories
   A. upregulated genes (Table 1)
   B. downregulated genes (Appedix A Table A.3)
3. Specific gene expression (Figure 11-13)

Figure 7. Microarray Flow Chart. Human peripheral eosinophils were isolated using negative selection, and treated for 4 h at 37°C with/without IFN-γ. After RNA isolation from each sample, RNA were labeled and hybridized to microarray. After microarray data was collected, single array analysis and comparison analysis were done using GCOS software. The data were further analyzed using GeneSpring and NetAffx.

CHAPTER III

Gene response to short-term IFN-γ treatment

3.1. RNA quality and quantity measurement by Agilent Bioanalyzer

Agilent LabChip PicoChip was used to test the quality and quantity of total RNA isolated from eosinophils with or without IFN-γ treatment. Initial results showed that RNA samples from eosinophils were rapidly degraded. Different optimization protocols were, therefore, used to achieve better quality. During optimization, it was also observed that eosinophil RNA samples were heavily contaminated with genomic DNA. This was observed as a peak emerging between the 18S and 28S peaks (Figure 8) and affected the purity of RNA samples. To remove genomic DNA contamination from samples, on-column DNAse I (Qiagen) treatment was incorporated to the RNA isolation protocol (Qiagen). Addition of this DNAse I step increased the quality of the RNA samples significantly (Figure 9). As well, isolation of RNA from a large number of cells (~10 x 10^6) using Qiagen RNeasyMini kit yielded the most amount of RNA (sample 1, Figure 9). Based on the result from Figure 9, a few modifications to the isolation of RNA protocol were made and RNA for 0min (control), 30min (IFN-γ), 4hr (control), and 4hr (IFN-γ) were isolated and pooled accordingly. The result of Agilent PicoChip analysis of these samples is shown in Figure 10.

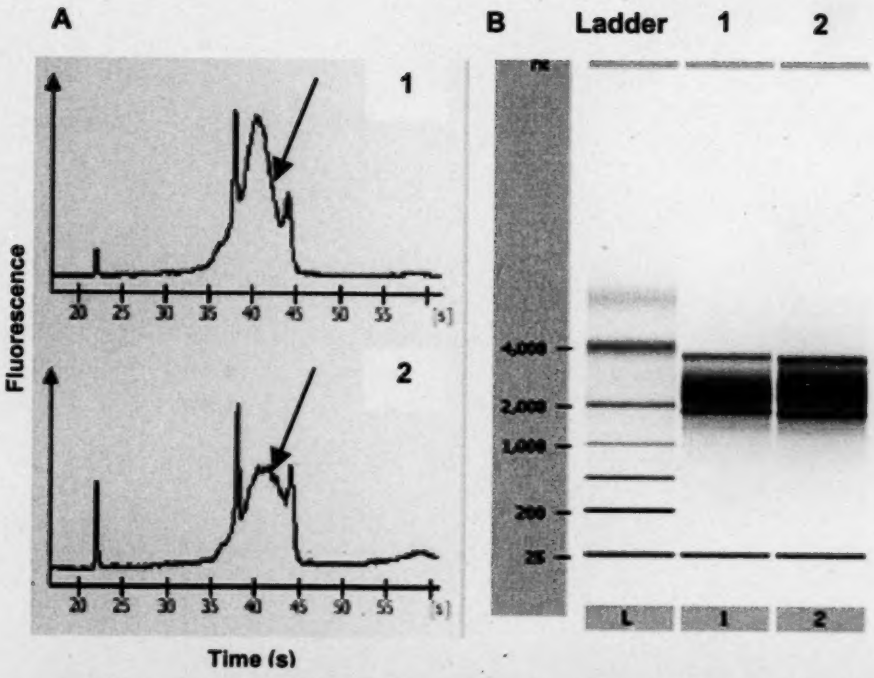
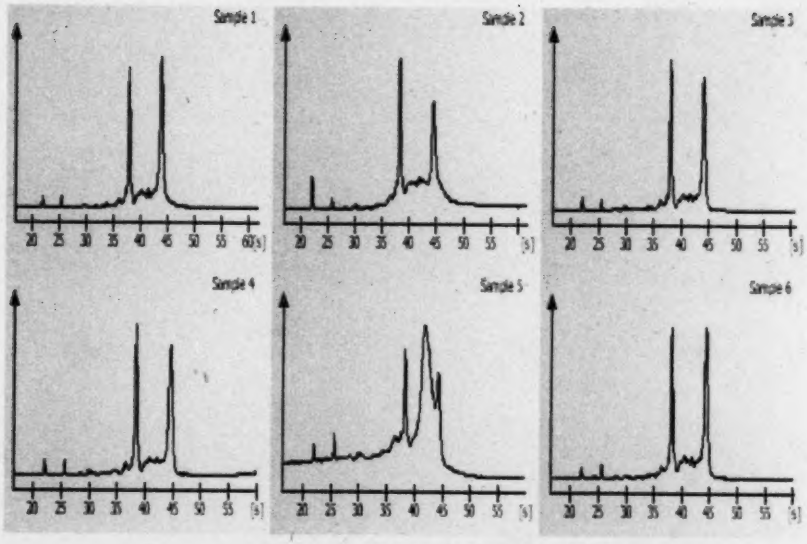


Figure 8. Total RNA samples from human peripheral eosinophils contaminated with genomic DNA. RNA was isolated according to the RNA isolation protocol (Qiagen) and was tested for the quality by using RNA 6000 LabChip. Data are displayed as A) electropherograms (samples 1 & 2) as well as B) a gel-like image. The arrows indicate contamination with genomic DNA. The RNA 6000 ladder (first lane) is used as a reference. Sample concentration and 18S/28S ratio: Sample 1. 11ng/µl, 0.8, Sample 2. 4ng/µl, 0.7

A


Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6

B

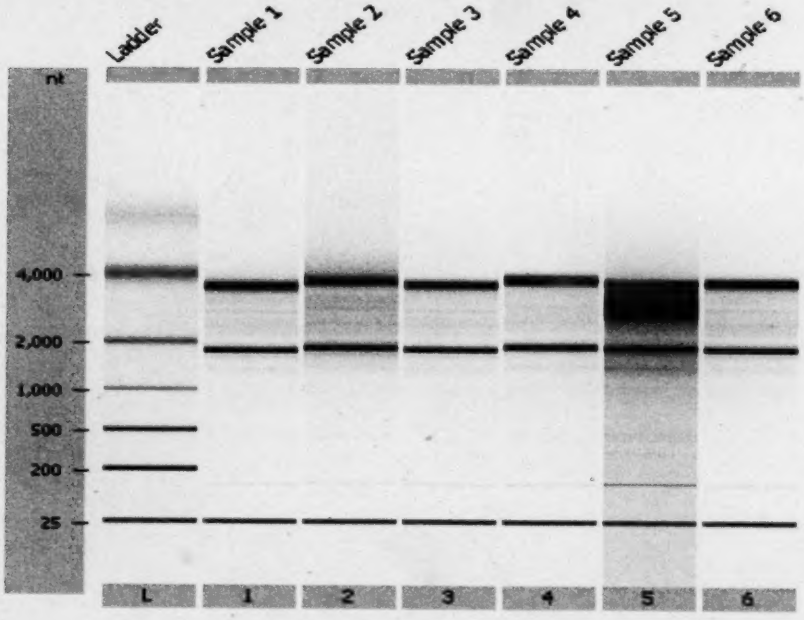
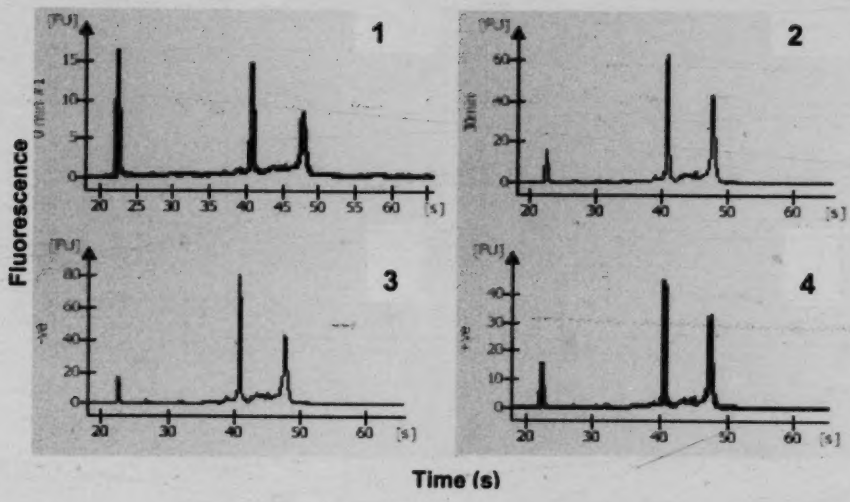

Ladder
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
nt
4,000
2,000
1,000
500
200
25
L
1
2
3
4
5
6

Figure 9. Assessment of quality of total RNA samples by RNA 6000 LabChip. Total RNA samples from human peripheral eosinophils was isolated and tested for their quality by using RNA 6000 LabChip. Each sample (1-6) varied in cell numbers or treatment conditions (Sample 1: 10 x 10^6 cells + Qiagen RNeasyMini kit + DNAse I treated. Sample 2: 7 x 10^6 cells + Qiagen RNeasyMini kit + DNAse I treated. Sample 3: 5 x 10^6 cells + Qiagen RNeasyMini kit + DNAse I treated. Sample 4: 5 x 10^6 cells + Qiagen RNeasyMicro kit + DNAse I treated. Sample 5: 5 x 10^6 cells + Qiagen RNeasyMicro kit + no DNAse I. Sample 6: 10 x 10^6 cells + Qiagen RNeasyMicrokit + DNAse I treated). Sample concentration and 18S/28S ratio; Sample 1: 4.2 ng/µl, 1.5, Sample 2: 2.6 ng/µl, 1.0, Sample 3: 2.0 ng/µl, 1.2, Sample 4: 1.9 ng/µl, 1.2, Sample 5: 6.0 ng/µl, 0.3, Sample 6: 2.5 ng/µl, 1.4. Data are displayed as A) electropherograms as well as B) a gel-like image. The RNA 6000 ladder (first lane) is used as a reference.

A



B

1. 0min control: R=1.0, RNA 0.5µg
2. 30min + IFNγ: R=1.3, RNA 1.2µg
3. 4hr – IFNγ: R=0.9, RNA 1.4µg
4. 4hr + IFNγ: R=1.2, RNA 0.8µg

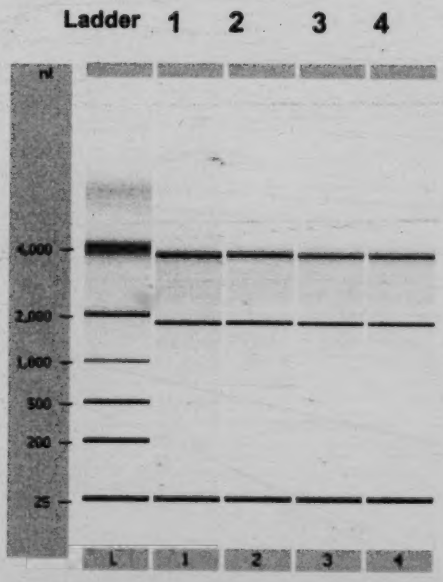


Figure 10. Assessment of final RNA samples by RNA 6000 LabChip. RNA samples from 10 donors were isolated, pooled together, and analyzed for quality and quantity using RNA 6000 LabChip. Data are displayed as A) electropherograms as well as B) a gel-like image. The RNA 6000 ladder (first lane) is used as a reference.

3.2. Detection of IFN-γ induced gene expression by Affymetrix GeneChip

Based on the Agilent PicoChip analysis, a high-density oligonucleotide probe array (Affymetrix GeneChip) was used to evaluate the expression of approximately 54,000 different mRNA transcripts in purified human peripheral eosinophils incubated for 4 h in the presence or absence of IFN-γ (200 U/ml). Subsequently, data from control and IFN-γ-treated samples were compared to detect changes in gene expression as diagrammed in the microarray flow chart as described in the Materials and Methods section (Figure 7).

The genes were considered upregulated when the corresponding fold increase was greater than or equal to 2.0 and downregulated when the corresponding fold decrease was less than or equal to -2.0. In this thesis, however, only the genes that had the corresponding fold change by at least 3 fold are listed to indicate robust changes (Appendix Table A.1 & A.2). The expression of 697 up-regulated genes, 775 down-regulated genes and ESTs, out of approximately 54,000 unique sequences, changed by at least 3 fold in IFN-γ induced versus uninduced eosinophil pair-wise comparisons. After eliminating ESTs of unknown function, 244 genes (upregulated) and 250 genes (downregulated) remained that had associated GenBank IDs (Table A.1 & A.2). This thesis will focus mainly on genes that showed upregulated expression after 4 h stimulation with IFN-γ. Conversely, only a few genes with known functions were downregulated following a 4 h treatment with IFN-γ. The entire list of downregulated genes (3 fold and up) is presented in the

Appendix A (Table A.2) to this thesis. The most prominently downregulated genes (among the top 100) with known functions are listed in Table A.3 of the Appendix.

3.3. Genes most prominently up-regulated (among top 100) in IFN-γ-stimulated eosinophils

The top 100 most upregulated genes were grouped according to their known functions (top 100 genes minus genes of unknown function) under several specific but not mutually exclusive categories (Table 1). These categories were created using tools such as GeneSpring software and NetAffx (*www.affymetrix.com*). The categories include transcripts associated with signal transduction, gene transcription regulation, immune response, apoptosis, transport mechanism, cell metabolism, cell adhesion and apoptosis. Among the genes undergoing the most prominent alteration in expression is serine (or cysteine) proteinase inhibitor (C1 inhibitor) (97-fold), which has been identified as a regulator of complement activation (Table 1) [91].

3.3.1. Transcripts associated with signal transduction

This category can be broadly classified into two; elements involved in G protein coupled receptor protein signaling pathway and elements associated with protein phosphorylation/dephosphorylation. Adrenergic (β-1) receptor and phosphodiesterase, associated with G-protein-coupled receptor pathway, were upregulated by 39-fold and 11-fold, respectively (Table 1).

Proteins associated with phosphorylation and dephosphorylation include BMX non-receptor tyrosine kinase (11-fold), serine/threonine kinase (10-fold) and dual specificity phosphatase 3 (9-fold). Rho guanine nucleotide exchange factor (GEF) 3, a transcript that does not belong to either sub-categories, was up-regulated by 9-fold and is implicated in Rho protein signal transduction.

3.3.2. Transcripts associated with immune/inflammatory response

Several other transcripts detected most prominently in RNA after 4 h of IFN-γ stimulation are genes that are involved in immune/inflammatory response including Toll-like receptors (TLR) 7 (9-fold) and 8 (24-30 fold) and all the currently known isoforms (1-5) of guanylate binding protein (GBP) (9-17 fold) (Table 1) (Figure 11). These genes are described in detail in section 4.2 and again in 4.5. This category also features CXCL9 (12-fold), also known as Mig, a chemokine that is thought to have a role in T cell trafficking, and is an IFN-γ inducible gene in macrophages [92].

3.3.3. Transcripts associated with apoptosis

Table 1 shows transcripts associated with apoptosis. Apoptosis is the result of a proteolytic cascade of cysteine proteases (caspases) which leads to cleavage of important substrates and subsequent cell death [93]. Many of the proteins involved in apoptosis are caspases and members of the tumor necrosis factor (TNF) family. In the current arrays, 4 h incubation with IFN-γ resulted in a 9-13 fold increase in TNFSF10, a soluble TNF-like molecule involved in the induction of apoptosis following ligation of its receptor [94].

Table 1. Upregulated genes (among the top 100) in eosinophils after IFN-γ stimulation (4hr) grouped according to their known biological functions

GenBank ID	Gene description	Fold Increase
Genes involved in signal transduction		
AI625747	Adrenergic, beta-1-, receptor	39
NM_001721	BMX non-receptor tyrosine kinase	11
L35594	Phosphodiesterase 2	11
NM_006281	Serine/threonine kinase 3	10
BC002682	Dual specificity phosphatase 3	9
NM_019555	Rho guanine nucleotide exchange factor (GEF) 3	9
Genes involved in regulation of transcription		
AF218365	ets variant gene 7 (TEL2 oncogene)	21
AI279555	checkpoint suppressor 1	15
NM_144657	hypothetical protein FLJ30678	13
AF147782	ets variant gene 7 (TEL2 oncogene)	12
NM_024509	hypothetical protein MGC2656	11
Genes involved in immune response/inflammatory response		
NM_000062	Serine (or cysteine) proteinase inhibitor (C1 inhibitor)	97
AW872374	Toll-like receptor 8	30
NM_016610	Toll-like receptor 8	24
NM_016562	Toll-like receptor 7	9
AW014593	Guanylate binding protein 1, IFN-inducible	17
BG271923	Guanylate binding protein 5	15
AL136680	Guanylate binding protein 3	15
BG545653	Guanylate binding protein 5	11
BF509371	Guanylate binding protein 2, IFN-inducible	9
BG260886	Guanylate binding protein 4	9
X14355	Fc frag of IgG, high affinity Ia, receptor for (CD64)	23
L03419	Fc frag of IgG, high affinity Ia, receptor for (CD64)	21
AI608902	Programmed cell death 1 ligand 1	15
NM_002416	Chemokine (C-X-C motif) ligand 9	12
NM_015364	Lymphocyte antigen 96	11
NM_005849	Immunoglobulin superfamily, member 6	11

Genes involved in apoptosis/immune response		
AF111345	Caspase 10, apoptosis-related cysteine protease	11
AW474434	TNF (ligand) superfamily, member 10	13
U57059	TNF (ligand) superfamily, member 10	10
NM_003810	TNF (ligand) superfamily, member 10	9
AI806486	Myeloid cell leukemia sequence 1 (BCL2-related)	9
Genes involved in transport mechanism		
AL833343	Potassium channel, subfamily K, member 1	23
AF323540	Apolipoprotein L, 1	17
AF305226	Apolipoprotein L, 4	9
BE550599	Calcium channel, voltage-dependent, L type, alpha 1D subunit	9
BF111326	Potassium inwardly-rectifying channel, subfamily J, member 2	9
Genes involved in adhesion		
NM_014893	Neuroligin 4	20
AA916861	C-type lectin protein CLL-1	16
Genes involved in metabolism		
AF323540	Apolipoprotein L, 1	17
BF666293	Follicular lymphoma variant translocation 1	12
AI830490	Glycerol kinase	10
BE788984	Aldose 1-epimerase	9
AI690583	Aspartyl aminopeptidase	9
Other Genes		
NM_020353	Phospholipid scramblase 4	49
NM_014917	Netrin G1	37
AA047234	Ornithine decarboxylase antizyme inhibitor	21
N25429	high-mobility group 20B	20
BC000606	Ribosomal protein L14	16
NM_006634	Vesicle-associated membrane protein 5 (myobrevin)	10
BC032854	Zuotin related factor 1	9

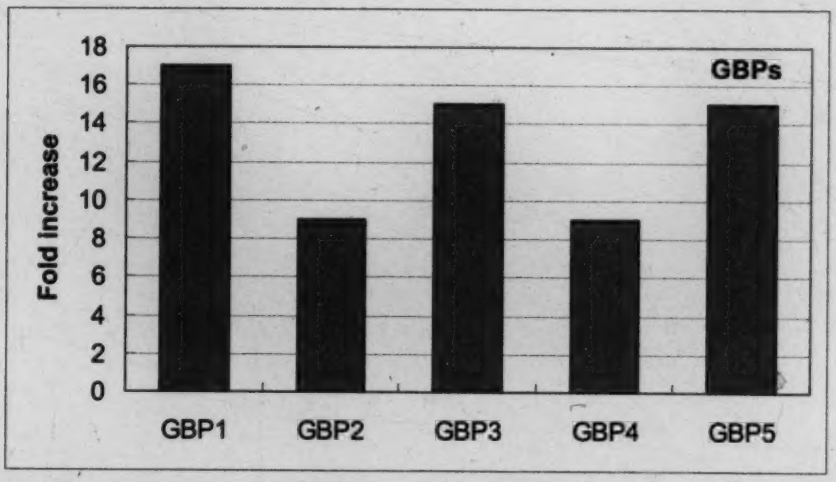


Figure 11. Analysis of guanylate binding protein (GBPs) gene expression in IFN-γ induced eosinophils. The prominent fold change, ranging from 9 to 17 fold, is observed for all GBPs (isoform 1 to 5).

Also, caspase 10, an apoptosis-related cysteine protease, whose gene expression has not previously been shown to be upregulated by IFN-γ was found to be upregulated (11-fold) in eosinophils.

3.3.4. Transcripts associated with cellular transport

Ion transport channels such as L-type calcium channel (9-fold) and potassium channel, subfamily K (23-fold) were also upregulated by IFN-γ. In addition, this category features new ISGs, apolipoprotein L 4 (APOL4) (9-fold) and apolipoprotein L 1 (APOL4) (17-fold), that are known to be involved in lipid transport [95].

3.3.5. Transcripts associated with metabolism and adhesion

Apolipoprotein L 1 is also included under genes involved in metabolism for its role in lipid/cholesterol metabolism, a role identified using GeneSpring software. In the same category, genes that are involved in carbohydrate metabolism, namely, glycerol kinase (10-fold) and aldose 1-epimerase (9-fold) were elevated. Also, gene expression for neuroligin 4 and C-type lectin protein CLL-1 that are involved in cell adhesion were elevated 20-fold and 16-fold, respectively (Table 1).

3.3.6. Transcripts associated with regulation of transcription

Using GeneSpring software, the following genes were denoted as the DNA-dependent regulators of transcription: ETS variant gene 7 (21-fold), hypothetical protein FLJ30678 (13-fold), MGC2656 (11-fold), and checkpoint suppressor 1 (15-fold) (Table 1).

3.3.7. Transcripts with unrelated functions

A few other genes that have no related functions were also included in Table 1. Netrin G1, for example, shown to be highly elevated in its gene expression (37-fold), is implicated in axogenesis. Phospholipid scramblase 4 (PLSCR4), which has a putative role in blood coagulation and in plasma membrane scrambling of phospholipids [96] is one of the most prominently upregulated gene (49-fold) in IFN-γ-induced eosinophils (Table 1).

3.4. Further analysis of genes involved in vesicular transport and innate immunity

Eosinophils have long been regarded as potent effector cells in parasitic infection and allergic inflammation. It is, therefore, interesting to examine the effect of a short-term IFN-γ stimulation on the expression of genes associated with intracellular transport leading to exocytosis. Similarly, changes in the expression of genes associated with innate and adaptive immune response were further examined. Although not prominent on the list among the top up-regulated genes (Table 1), there was an increased expression of the transcript, VAMP5 (10-fold). This protein has been implicated in myogenesis (cellular differentiation) [97]. Conversely, two VAMP isotypes, namely VAMP2 and VAMP10, were found to be downregulated by 3-fold and 10-fold, respectively, following short-term treatment with IFN-γ (Figure 12). Other than VAMP5, no isotype of VAMP was seen upregulated by more than 3-fold.

In addition, transcripts for TLR7 and 8 were upregulated after a 4 h

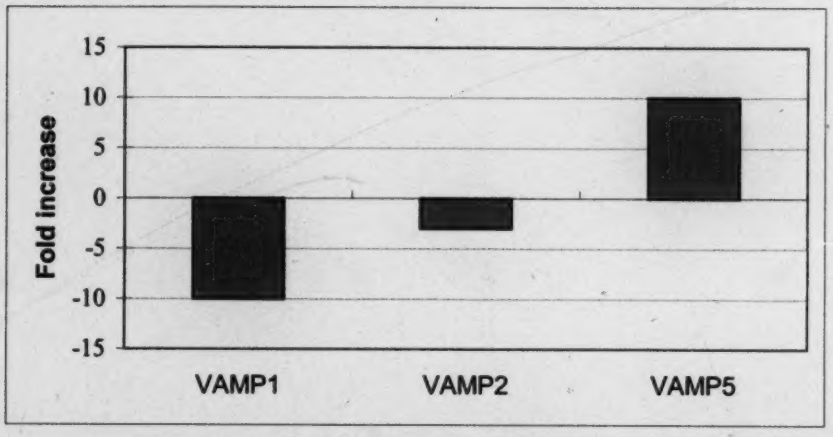


Figure 12. Analysis of VAMP gene expression in IFN-γ induced eosinophils. VAMP1 and 2 were downregulated by 10-fold and 3-fold, respectively, whereas VAMP5 gene expression was upregulated by 10-fold.

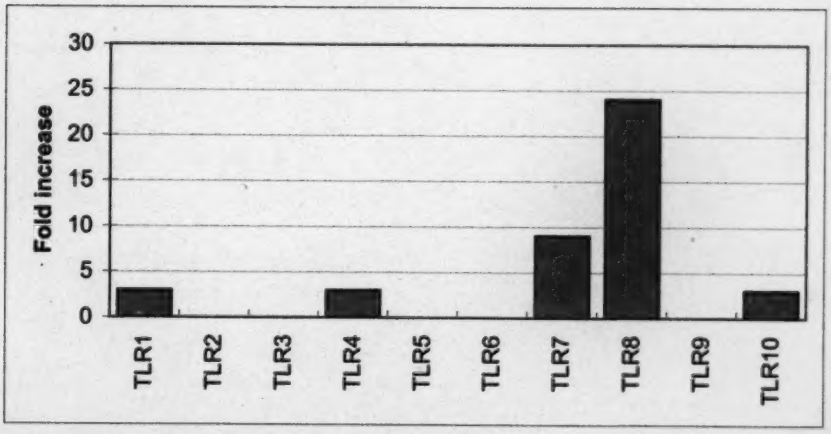


Figure 13. Analysis of Toll-like receptor (TLRs) gene expression in IFN-γ induced eosinophils. The transcripts for TLR1, TLR4, TLR7, TLR8 and TLR10 were upregulated after IFN-γ stimulation.

treatment with IFN-γ. Other isoforms of TLR transcript (TLR1, TLR4, TLR10) were also up-regulated (3-fold) in IFN-γ-treated (Table 1) (Figure 13).

In IFN-γ-treated eosinophils, several interferon-gamma-inducible genes were also found to be upregulated. Among these, transcripts involved in immune response, such as CXCL 9 (12-fold), CXCL-11 (8-fold), (CXCL9 and CXCL11 chemoattractant for monocytes and T cells) [98] and IDO (3-fold), were identified (Appendix Table A.1).

3.5. Validation of Affymetrix GeneChip data

3.5.1. Real-time PCR for IDO

We first investigated whether IDO is expressed in eosinophils obtained from atopic donors. This work was recently published from our laboratory [44]. Using primer pairs designed to amplify a 201-bp fragment of IDO (primer set 2), both untreated and treated (4 h with IFN-γ) eosinophils were found to express IDO-specific transcripts (Figure 14). Subsequently, IFN-γ-induced IDO mRNA expression in eosinophils was measured using quantitative real-time RT-PCR with primer set 1 (230-bp) (Figure 15). A standard curve was generated following amplification of serially diluted gel-purified IDO cDNA standards with known gene copy numbers (10^8-10^3 copies). Figure 16 shows the melting curve of the amplified standards. Specific products melted between 85-93°C. Plotting the normalized fluorescence against the cycle number allowed the determination of threshold fluorescence for each dilution of the standard (C_t values) (Figure 17). To

generate a standard curve, C_t values were plotted against copy numbers (Figure 18). For absolute quantification of RNA in IFN-γ-stimulated eosinophils, normalized copy number (normalized with 18s rRNA) was read off the standard curve (Figure 15). We observed ~ 4-fold induction of IDO mRNA expression in IFN-γ-stimulated eosinophils (Figure 15). Similarly, our microarray analysis indicated 3-fold increase in IDO transcript.

3.5.2. Real-time PCR results for TLR4, TLR7 and TLR8 transcripts

A publication by Hirai and colleagues also validated our microarray data [51]. As part of their study, this group incubated human peripheral eosinophils with IFN-γ for 4hr at 37°C, isolated RNA using the Qiagen RNeasy Kit and studied TLR gene expression by real-time RT-PCR. Three TLR genes were found to be upregulated, namely, TLR4, TLR7 and TLR8 [51]. Our microarray data has confirmed this observation (Figure 13). Indeed, TLR4 mRNA expression was shown to be increased 2- to 3-fold by IFN-γ [51], which is similar to our microarray result for TLR4, which showed a 3-fold increase in its gene expression after IFN-γ stimulation (Appendix A.1). In addition, they observed the most prominent increase in both TLR7 (~10 fold) and TLR8 (~10 fold) gene transcripts [51], which is also in agreement with our microarray analysis (Figure 19). Moreover, this group showed very little TLR7 and TLR8 mRNA expression prior to IFN-γ stimulation (Figure 19) [51]. In our hands, TLR7 and TLR8 gene transcript were also negligible in the control (untreated) when the single array analysis was performed (data not shown).

Figure 14. IDO gene expression in human eosinophils by RT-PCR analysis of IDO mRNA expression. IDO expression was detectable in nonstimulated (Donor #1) and IFN-γ-treated human eosinophils (Donor #2). #1: donor 1, #2: donor 2, 18s rRNA was used as a control.

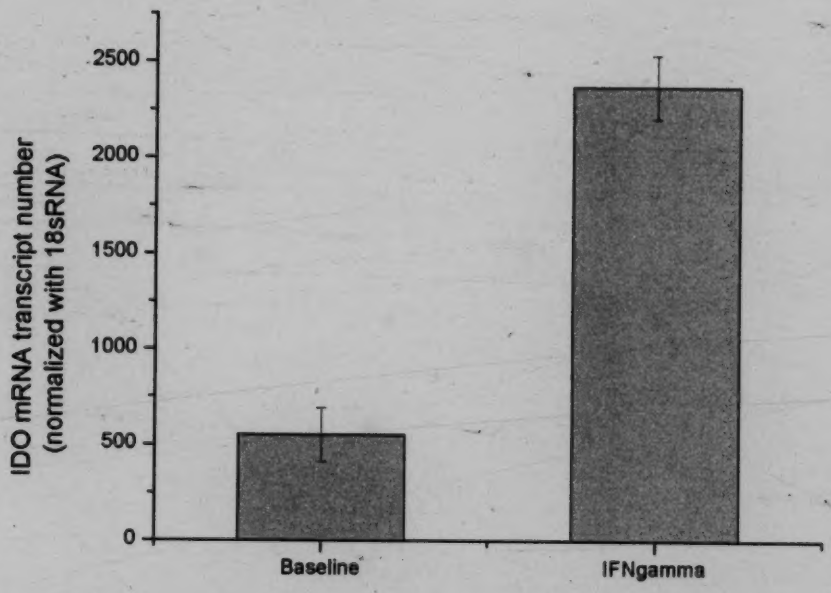


Figure 15. Real time-PCR analysis of IDO mRNA expression. Following treatement with IFN-γ for 4 h at 37°C, RNA was isolated. Following reverse transcription, PCR was performed with IDO-specific and 18S ribosomal RNA primers and quantified using SYBR Green I dye. A standard curve was generated following the amplification of known starting copy numbers of IDO and 18S rRNA. Copy numbers of IDO mRNA were determined following normalization of the mRNA concentration with 18S rRNA (generated by S. Odemuyiwa (n=4) and J. Lee (n=2))

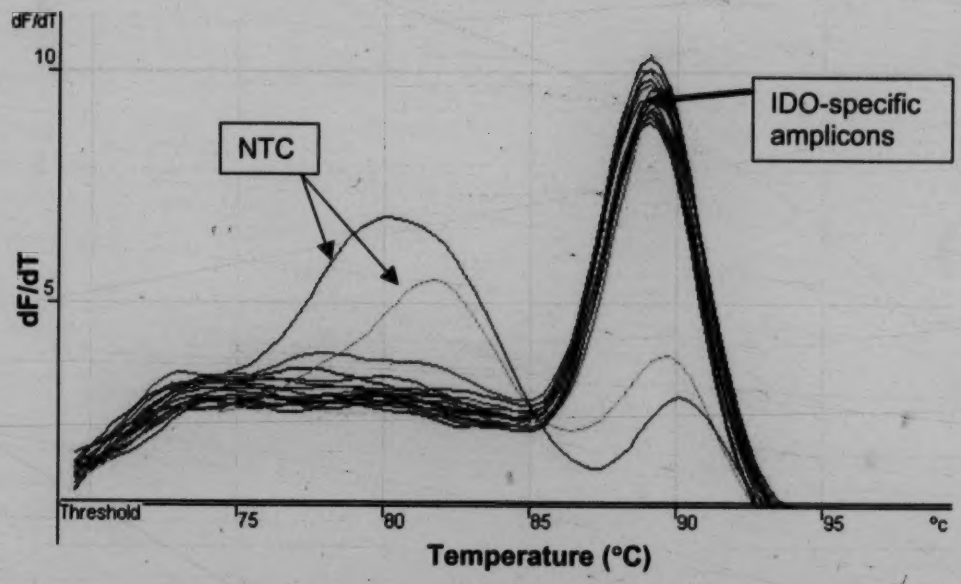


Figure 16. The melt curve analysis of IDO-specific amplicons. This analysis shows melting of amplification products at the end of a real-time PCR run. As part of SYBR Green optimization, this curve is used to differentiate between target and artifact signal (such as primer-dimers and other non-specific reaction products). The melting temperature (Tm) for IDO-specific amplicons is 89°C. Primer-dimers in no-template control (NTCs) melted between 75-85°C. Once this is obtained, the temperature profile in the machine is adjusted so that data can be acquired above the Tm of primer-dimers and below the Tm of IDO-specific amplicon to make the primer-dimers disappear.

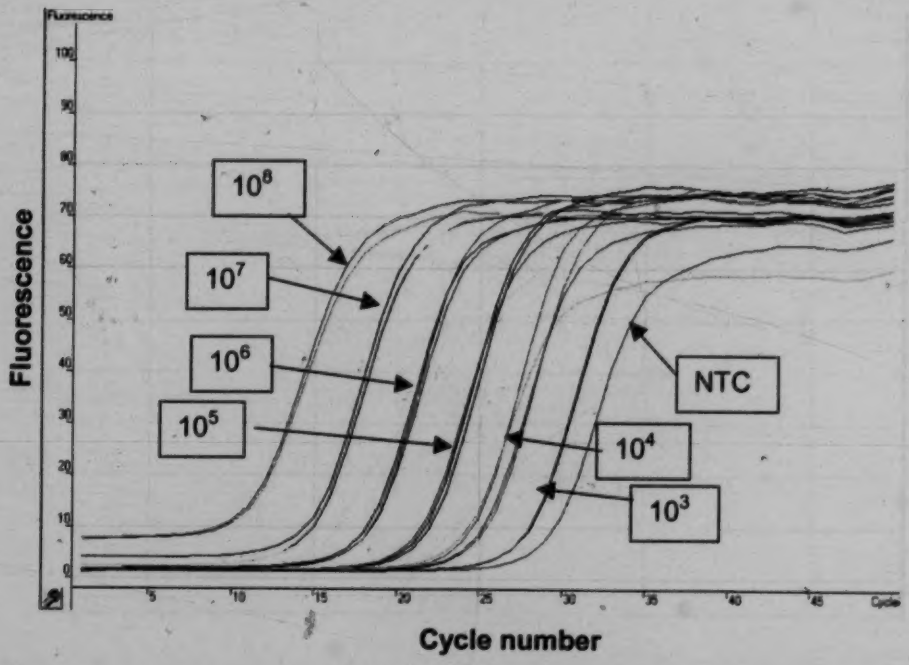


Figure 17. Rotor-gene raw data representing a dilution series of triplicate reactions for IDO. The dilutions were made according to the copy numbers of DNA calculated from gel-purified IDO product. The dilutions ranging from 10^3 to 10^8 copies of IDO DNA were plotted by the normalized fluorescence against the cycle number. This allowed the determination of threshold flurorescence for each dilution of the standard (C_t values). This graph is a necessary step to generate a standard curve from which the copy numbers of the samples can be calculated. *C_t value:* number of cycles for a sample to reach the threshold level where the rate of amplification is the greatest during the exponential phase. NTC: no-template control.

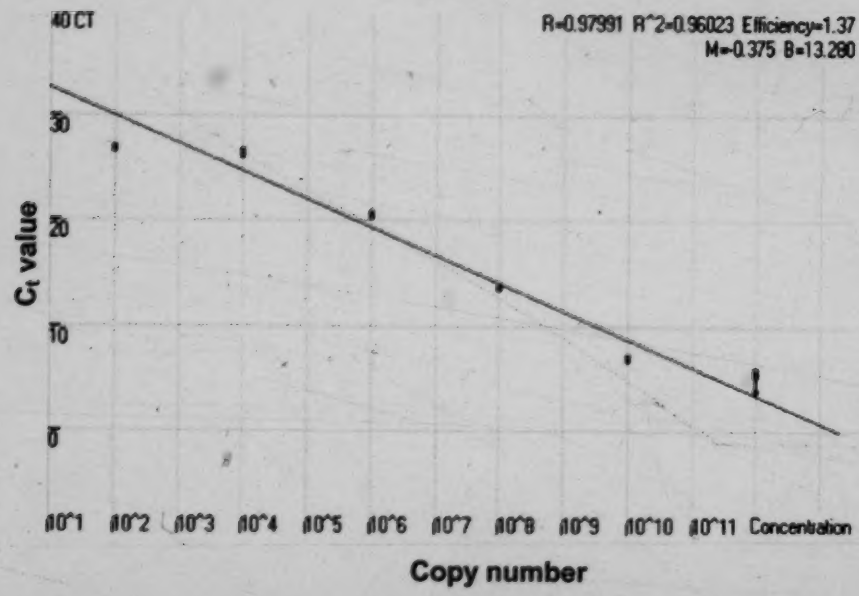


Figure 18. The standard curve has been taken to obtain the absolute copy number of 18S rRNA. The top right corner shows the R-value, the slope and the intercept and efficiency. This was generated by plotting C_t values (y-axis) against copy numbers (x-axis). The R-value of this experiment is 0.97991 and the reaction efficiency is 1.37, where 1 is a 100% efficient reaction.

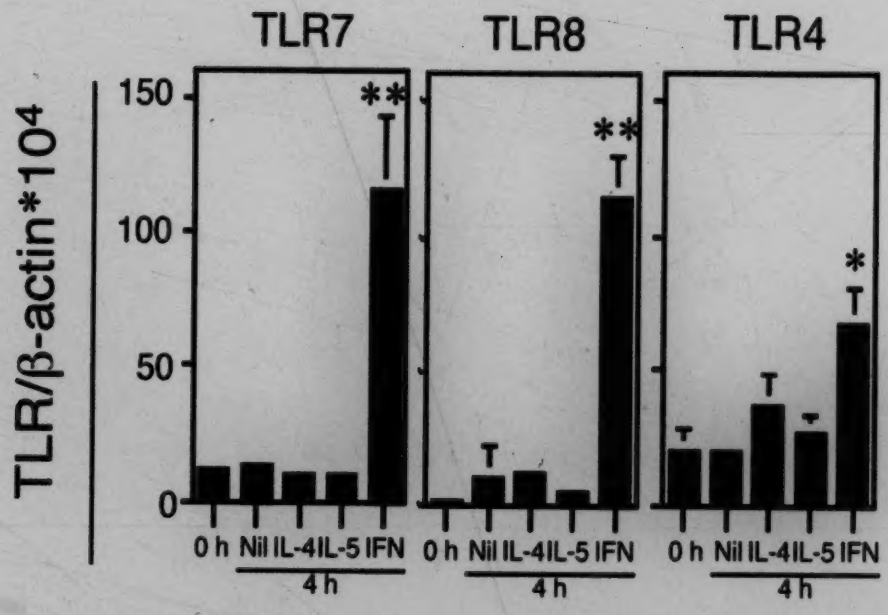


Figure 19. Modulation of expression and function of eosinophil TLR by cytokines. The level of TLR mRNA expression was determined by real-time PCR in eosinophils just after purification (0 h), or in cells cultured for 4 h with IL-4 (10 ng/ml), IL-5 (10 ng/ml), IFN-γ (300 U/ml) or medium alone (Nil). The data are expressed as a ratio: copy number of TLR gene/copy number of β-actin gene. ••, $p < 0.01$, •, $p < 0.05$ vs value of Nil ($n = 3$).
(Copied from: *Hirai et al., Journal of Immunology, 171: 3977-3982, 2003.* ***Copyright* 2003**. *The American Association of Immunologist, Inc.*)

CHAPTER IV

Discussion & Conclusion

4.1. Genes regulated by IFN-γ in the human eosinophil

There is paucity of information on the effects of IFN-γ on eosinophil gene expression. The present study examined the profiles of genes induced by IFN-γ in eosinophils following a relatively short-term stimulation. Using 4 h stimulation in the absence of IFN-γ as a baseline, a comparative analysis was performed to detect changes in gene expression. At least a three-fold up-regulation or down-regulation was found in approximately 700 genes and 800 genes, respectively. The functions of many of these genes with changes in expression are currently unknown. However, the functions of 244 upregulated and 250 downregulated genes have been previously characterized (Appendix Table A.1 & A.2).

It is important to emphasize that this study was focused solely on changes in gene expression detectable at 4 h post IFN-γ stimulation. This does not exclude the possibility that other genes may have also been either upregulated or downregulated at other time points of stimulation before or after 4h.

4.2. Genes of specific interest induced by IFN-γ in eosinophils

Several genes of specific interest have emerged from the microarray data. Most prominent was the profound increase in expression of genes

associated with immune/inflammatory response (Table 1). For example, 9- to 30-fold IFN-γ-dependent increases in the transcripts of genes encoding for TLR7 and TLR8 were observed. This finding is similar to the results of recent data from Hirai *et al.* using a real-time PCR to analyze changes in the expression of TLRs in eosinophils stimulated with IFN-γ [51]. This may suggest an important role for eosinophils in responses characterized by a copious production of IFN-γ. For example, eosinophils may be stimulated by this cytokine if present during a viral infection where IFN-γ is abundant. Interestingly, the microarray analysis also detected at least a 3-fold increase in the expression of TLR1, TLR4 and TLR10 (Figure 13). TLR4 mRNA has been found to increase following IFN-γ stimulation in eosinophils, however, the protein expression was not detected in eosinophils [51;52]. Similarly, whether the gene expression for TLR1 and TLR10 translate to functional proteins is not known and should be investigated.

Furthermore, with the microarray analysis, there is an increase in the expression of GBPs, strongly suggesting that eosinophils may play important roles in innate immune response. In IFN-γ-stimulated eosinophils, transcription of the genes encoding all of the currently known isoforms of GBPs was increased 9- to 17-fold (Figure 11). GBPs are IFN-inducible guanosine 5'-triphosphatases (GTPases) [99] that play a role in restricting the spread of viruses. Previously, IFN-γ has been shown to activate GBPs 1-5 in macrophages [99]. However, no studies have described the magnitude of gene expression of all GBP isoforms (1-5) in IFN-γ-stimulated eosinophils

until now. It would be interesting to investigate the role of GBPs in eosinophils stimulated with IFN-γ.

The same category also features the most prominent transcript encoding serine proteinase inhibitor (SERPING1) (97-fold). This profoundly increased level of SERPING1 expression is particularly intriguing since this has recently been suggested to play an anti-inflammatory role by down-regulating leukocyte migration from the vasculature during inflammation [100]. Some studies have shown that IFN-γ-treated hepatocytes, monocytes, endothelial cells, or fibroblasts have increased expression of SERPING1 (C1 inhibitor) mRNA, which leads to the synthesis of C1 inhibitor protein [101]. Thus, the high level of increase in the expression of this gene observed in this study suggests that eosinophils may have roles related to complement function.

Several other IFN-γ-inducible genes were also identified in the microarray. Prominent among these are, PLSCR4, CXCL9 (Mig), CXCL11, and IDO. IDO has recently been shown to be an important immunomodulatory protein [44]. It is constitutively expressed in eosinophils and the gene expression is upregulated in the presence of IFN-γ [44]. A finding of constitutive expression as well as increased transcript of IDO in our microarray data confirms our previous findings.

Phospholipid scramblase is another gene that is recently described as a IFN-γ-stimulated gene. In our microarray analysis, PLSCR4 gene expression was very highly upregulated (49-fold) (Table 1). Interestingly,

another member of this family, PLSCR1, has recently been implicated in anti-viral [102] and apoptotic functions [93]. As such, PLSCR1 has been postulated to contribute to the antiviral effects of IFN-γ by affecting viral penetration, the transcription of antiviral genes in the nucleus and by directly blocking specific stages in the viral replication cycle [102]. Although not well characterized yet, PLSCR4 may also have potentially more complex roles, related to eosinophils, beyond its putative role in mediating transbilayer lipid movement [96].

G protein-coupled receptors (GPRs) and ion channels are increasingly being recognized for their importance due to their essential roles in degranulation and other related functions for granulocytes [103]. As a result, these molecules are potential targets of drug development. In eosinophils, IFN-γ-induced the up-regulation of adrenergic (β-1) receptor expression and calcium (L type) and potassium channels.

One of the major interests in our lab is on the mechanism of exocytosis in eosinophils. Among the molecules involved in exocytosis, SNAP receptors (SNAREs) are small membrane-associated proteins involved in distal docking of membrane-bound transport during vesicle/granule docking with the target membrane [104]. Previously, these proteins have been reported to localize to either the vesicle or presynaptic membrane in neurons, participating in the release of neurotransmitters [105]. SNARE complex proteins consist of two functionally distinct classes of SNAREs, vesicular (v-) and target membrane (t-)-SNARES [106]. In case of

synaptic vesicle exocytosis, VAMP-2 is a v-SNARE and syntaxin and SNAP25/23 are t-SNARE [104]. Our studies have recently identified that cells outside the central nervous system also express VAMP-1 and VAMP-2 [107].

Previously, our lab detected VAMP-2 mRNA expression in eosinophils [107]. Rapid mobilization of VAMP-2 with RANTES during IFN-γ was associated with eosinophil piecemeal degranulation [107]. Intriguingly, our data from microarray analysis showed a 3-fold down-regulation of VAMP-2 transcripts in IFN-γ-induced eosinophils (Figure 12). On the other hand, VAMP-1 transcript was down-regulated 10-fold following IFN-γ stimulation. In an earlier study, VAMP-1 could not be detected in guinea pig eosinophils [108]. Conversely, however, there is an increased transcription of VAMP-5 (10-fold) following IFN-γ stimulation. VAMP-5 is implicated in non-selective vesicle transport [109] and myogenesis [97]. The majority of VAMP-5 appears to be distributed at the most distal (plasma membrane) parts of the post-Golgi structures [110]. More work is needed to decipher its role in eosinophils.

The outcome of the observations for my study is the pair-wise comparison analysis of Affymetrix data which may suggest several testable hypotheses regarding eosinophil immunobiology and the capacity of its transcriptomes in various aspects of eosinophil function. It has also provided specific insight into the nature of the IFN-γ response in eosinophils towards future research and potential novel therapeutic strategies.

4.3. Microarray studies; potential technical pitfalls

When designing a microarray study, it is important to be aware of technical variability that may arise within the system. Sources of variability come from biological system, sample preparation (total RNA isolation as well as labeling), and technical factors (instrument and arrays) [82].

As a result of standardization of steps including hybridization, washing, staining and scanning, as well as the quality controls built into manufacturing processes, system noise (instrument and arrays) does not provide a significant source of technical variation (Affymetrix GeneChip Expression analysis). On the other hand, a major source of variation may come from sample preparation. As for the initial isolation of RNA step, care should be used to ensure that the RNA is of high quality and consistently suitable for labeling and array hybridization [82]. Several aspects of labeling should also be controlled to minimize process variability including processing all RNAs on the same day, using reagents from the same lots, preparing reagent master mixes, and having a single scientist responsible for all the bench manipulations [82].

There is also variability associated with the fragmented cRNA length (35 to 200 base) used in Affymetrix GeneChip. Some of the longer sample fragment sizes will have a reduced transcriptional efficiency compared with shorter sequences. This may have an effect on the accuracy in detecting gene expression.

Another source of variability may arise from the biological system.

Homogenous cell populations such as cell lines or purified cell populations are more likely to represent gene expression levels in the study [81]. Conversely, tissue or organ samples may add variabilitiy to microarray studies as these sources represent a diverse cell population. Therefore, gene expression profiles obtained from tissue or organ samples may not represent the precise experimental conditions [81]. It is, therefore, important that these variables should be controlled carefully to avoid highly variable or un-interpretable data.

4.3.1. Microarray studies in the eosinophil; technical considerations

In order to obtain the best microarray gene analysis, isolation of high quality and quantity of RNA are desired. In this respect, eosinophils offered several unique and at times frustrating obstacles. Eosinophils are end-differentiated granulocytes that express low concentrations of RNA when unstimulated [1]. In addition, they contain highly potent RNAses (EDN, ECP) in their crystalloid granules [111]. RNAse-rich granules derived from lysed cells rapidly destroy RNA transcripts. These factors contribute to limited RNA sample quantities and poor RNA quality. In our experiment, several modifications to the protocol were made to improve the quality of isolated RNA. As for improving quantity, similar to other microarray studies in eosinophils [85;112], the sample pooling method was employed to obtain adequate amount of RNA samples.

Pooling of samples can be a crucial source of variation and should only be done when it is impossible to obtain enough RNA otherwise, since

sample pooling may result in irreversible loss of information (Affymetrix GeneChip Expression analysis). Once RNA samples are mixed, there is no way to identify whether any one sample was a biological outlier [81]. In addition, mixing samples may prevent the detection of small magnitude changes that are reliable and may be biologically important. Therefore, one should be aware of these limitations when interpreting data obtained from pooled RNA samples.

In our microarray study, it was necessary to pool RNA samples from 15-20 asthmatic/allergic donors. Our RNA extraction method using Qiagen RNeasy mini kit yielded approximately 0.1 μg of RNA per donor per treatment, which was not enough for the subsequent labeling step (one-cycle target labeling, Affymetrix. CA) (Figure 6). Although two-cycle target labeling renders labeling as little as 10 ng of samples possible (Affymetrix. CA), this option was not feasible to us due to its high cost. Also, obtaining RNA from the same donor repeatedly found to be challenging given many factors (frequency of donation, volume of blood per donation). Taken together, it was necessary, and maybe effective and useful, to pool eosinophil RNAs from donors despite the above listed limitations associated with sample pooling in microarrays.

4.4. Real-time RT-PCR supporting the microarray data

Four of the genes that were screened using the microarray technique were verified with real-time RT-PCR that has much higher sensitivity of the

amplification method. Although the verification of three other genes, other than IDO, came from real-time RT-PCR studies by another group, the experimental conditions used (stimulus, human donors, time of stimulation, eosinophil isolation method, and RNA extraction method) were strikingly similar to that of my microarray study.

Nevertheless, one should be aware that some variations exist between the cDNA array and RT-PCR results due to the different primer sequences of the two methods. Regarding the sequence specificity, the cDNA array technique has been reported to give discrepant results in a portion of cases [113]. However, it remains to be the most suitable screening method because of the broad range of gene expression that can be detected. Although it is encouraging that the results of the cDNA array were confirmed by other studies, these findings require confirmation by a second or third set of pooled cDNA samples from eosinophils before the results can be validated appropriately.

4.5. Generation of hypotheses

Several testable hypotheses were generated as a result of the microarray analysis. In the following section, these hypotheses will be discussed.

4.5.1. TLRs

Respiratory viral infections trigger asthma/allergic exacerbation [54]. It

has been postulated that one of the mechanisms of asthma exacerbation is the involvement of eosinophils during viral infection. Respiratory viral infections in allergic/asthmatic airways are capable of producing a varied inflammatory response, including prominent airway eosinophilia and production of IL-5 by both CD8+ and CD4+ T cells [114]. Although under normal conditions, CD8+ T lymphocytes produce IFN-γ in response to viral infections, it has been shown that in an allergic milieu, CD8+ cells respond to viral infection by producing IL-5, thereby contributing to an eosinophilic response in the lung [114]. The influx of eosinophils in the airway may contribute to bronchial inflammation. Several studies also have indicated that eosinophils are recruited to and degranulate within the lung parenchyma during severe infections with respiratory syncytial virus (RSV) [111]. RSV infection leads to IFN-γ production, which may provide a mechanism for eosinophil activation. While it may be true that eosinophils cause damage in the airway by releasing harmful mediators, it is not well known how exactly these cells become activated once they arrive in the airway. The microarray results generated here indicate prominent up-regulation of TLR7 and TLR8 gene expression in IFN-γ-stimulated eosinophils. These data confirm previous work on TLR7 and TLR8 [51]. It is, therefore, conceivable that recognition of single stranded viral RNA through TLR receptors could potentially be a mechanism of IFN-γ-dependent eosinophil-mediated viral killing *in vivo*.

Eosinophils may both contribute to virus-induced asthma

exacerbations and play a beneficial role in antiviral response. Eosinophils store potent mediators such as EPO, ECP and EDN in their granules [12]. EPO has been shown to have antiviral activity [115]. ECP and EDN are recognized as members of the RNase A superfamily with potent ribonuclease activities that exert antiviral effects [54;116]. A study has shown that eosinophils effectively reduce viral infectivity via the actions of their secreted ribonucleases in RSV experimental system [116]. In the same study, it was found that EDN alone could function effectively as an antiviral agent [116]. Another study reported a similar finding with ECP [117]. It would be interesting to determine whether TLR7 and TLR8 activation by viruses leads to the release of EPO, EDN and ECP, all of which possess antiviral properties. IFN-γ does not induce significant release of these granule-derived mediators, but may actually enhance or prime eosinophils to degranulate in response to viruses. Ultimately, this could be an important mechanism linking viral infection and exacerbation of allergic disease.

Hypotheses: Based on my microarray result that confirmed TLR7 and TLR8 data generated by Hirai's group [51], I propose that *IFN-γ-stimulated eosinophils are activated by single-stranded RNA viruses through a direct interaction with their TLR7 and TLR8 during viral infection.* Furthermore, I also hypothesize that *eosinophils, following activation through interaction of these receptors, play an antiviral role by releasing mediators such as EPO, EDN and ECP.* Therefore, through the interaction with these receptors, eosinophils may be important in host-defense against viral

infections. Currently, TLR7 and TLR8 antibodies are not available and therefore, direct detection of the protein expression on eosinophils will not be feasible. However, chloroquine, an endosome acidification inhibitor shown to inhibit the activation of TLR7 and TLR8, could be used to test whether eosinophil-derived EDN and ECP have any effect on reducing viral infectivity. A dose-dependent decrease in RSV infectivity by EDN and ECP may be inhibited using various ribonuclease inhibitors to test a role for the eosinophil secretory ribonucleases.

4.5.2. Guanylate binding proteins (GBPs)

Among many newly described host proteins induced by IFN-γ with antimicrobial activity is guanylate binding protein (GBP). These proteins are 65-68kDa GTPases that exhibit robust GTPase activity [99]. In mice, five members of GBP have been identified to date and a similar number exist in the human system [99]. Except for GTPase activity *in vitro*, little information is available on the function of GBPs. Some studies have shown that hGBP1 mediates antiviral effects against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) in HeLa cells [118]. Also, the same protein was shown to exhibit anti-proliferative activity of endothelial cells [119]. This activity occurs in the absence of apoptosis and is independent of the GTPase activity of GBP-1. It is mediated specifically by the C-terminal helical domain of hGBP-1 [119]. The latter study suggested that the anti-proliferative activity may help limit the cell-to-cell spread of progeny virus

[119]. Other members, such as mGBP2 and mGBP4 mRNAs are strongly induced in the liver following *L. monocytogenes* infection [120]. The antibacterial effect of GBPs on *Listeria* has not been investigated [120]. Recently, mGBP2 was shown to inhibit the replication of both VSV and EMCV [121].

Unlike other GTPases, all GBPs are classical secondary response genes, relying on the *de novo* synthesis of transcription factors, such as IFN regulatory factor-1 (IRF-1) induced by IFN-γR signaling, via JAK-STAT-dependent pathways [99]. GBPs require IRF-1 to bind to interferon-stimulated response element (ISRE) for full expression. Studies have shown that at least the expression of GBP1 and GBP2 protein are cycloheximide-sensitive [120;122].

Since the induction of GBPs is common to many cell types that respond to IFN-γ, it is possible that GBPs may mediate IFN-γ-induced responses in inflammatory disease. In an experimental model of asthma, it was shown that virus-resistant rats develop acute airway inflammation that resolved rapidly following infection with the Sendai virus, when compared with virus-susceptible rats that developed chronic airway dysfunction [123]. Interestingly, mGBP-2 was 1 of 3 genes to show significantly higher expression in virus-resistant strain of rats [123]. This suggests that GBP may play an important role in the development of Th1 responses, rapid virus clearance, and resistance to virus-induced chronic airway inflammation in rats following Sendai virus infection.

Nothing is known about eosinophils and GBPs at present although eosinophils have been suggested to play a role in antiviral host defense. As shown for TLRs, eosinophils have been shown to mediate a dose-dependent reduction in virus infectivity with respiratory syncytial virus (RSV) [116]. It is, therefore, possible that GBPs may play a role in the ability of eosinophils, to combat virus infection in synergism with secreted eosinophil ribonucleases including EDN, and ECP.

Hypotheses: Given the importance of both eosinophils and GBPs in viral infections, I hypothesize that *GBPs play a critical role in the inhibition of respiratory viruses, for example, RSV, Rhinovirus, in eosinophils activated by IFN-γ.* The presence of GBP protein expression in eosinophils can be examined by Western blotting with 4 h IFN-γ stimulation. Eosinophils can be transfected with the human IFN-γ-induced GBP-1 to express GBP-1 constitutively. This population can then be compared to eosinophils producing a reduced amount of GBPs in response to IFN treatment as a result of the expression of an antisense RNA to hGBP-1. Subsequently, eosinophils can be infected with virus (RSV, Rhinovirus) and viral yield can be determined to see the virus-mediated cytotoxic effect.

4.5.3. Serine proteinase inhibitor, C1 inhibitor (SERPING1)

C1 inhibitor, a 104kDa glycoprotein, is a member of the serine proteinase inhibitor (serpin) family [124]. The primary biological role of C1 inhibitor is to regulate activation of the complement system. It also inhibits the

contact system of kinin generation by inhibiting several proteases including C1s, C1r, kallikrein and activated Hageman factor [124]. IFN-γ induces increased expression of C1 inhibitor in many cell types, including endothelial cells, monocytes and hepatocytes [101;124]. C1 inhibitor has been shown to downregulate leukocyte migration during inflammation by interfering with endothelial-leukocyte adhesion [100]. It is reasonable to assume, then, that C1 inhibitor present during inflammation has an anti-inflammatory role. Surprisingly, in our microarray study, C1 inhibitor was observed to be the most up-regulated gene (97-fold) in eosinophils stimulated with IFN-γ.

Currently, nothing is known about C1 inhibitor in eosinophils. It is possible that eosinophils may play an anti-inflammatory role during inflammation through the actions of C1 inhibitor. For many years, eosinophils were considered to be involved in the pathophysiology of allergic diseases, contributing to the destruction of normal tissue in inflammation. Recently, however, more recent studies have suggested that eosinophils may also play beneficial roles in the immune system such as anti-viral responses [111]. Through the actions of C1 inhibitor in counteracting the proteases released at sites of inflammation, eosinophils may contribute to tissue protection in the lung. In a number of inflammatory conditions including asthma, allergic rhinitis and inflammatory bowel disease, both neutrophils and eosinophils are present at the sites of inflammation [125]. Activated neutrophils are known to secrete serine proteases such as elastase that damage connective tissue matrix in the lung [125]. Elastase is inhibited by alpha-1-antitrypsin (AAT),

which has been shown to be present in human eosinophilic granules [125]. Although C1 inhibitor has been shown to have no inhibitory effect on elastase [126], a clinical study has suggested that C1 inhibitor may reduce activation of neutrophils in patients with sepsis [127]. The same group also suggested that the activation of neutrophils in septic patients occurred via IL-8 or the complement system [127].

During viral infection, a profound inflammatory response is triggered [111]. Neutrophils and eosinophils are recruited to the site of inflammation, with eosinophils showing up very early in time, and neutrophils infiltrating later [111]. In another inflammatory disease, ulcerative colitis, both neutrophils and eosinophils are implicated [128].

Hypotheses: Given all the findings above, it is possible that eosinophils, in the presence of IFN-γ, may upregulate the synthesis and release of C1 inhibitor. Most of C1 inhibitors are found in the plasma but local synthesis and release may be necessary at sites of inflammation. Once released, C1 inhibitor may attenuate the activation of neutrophils. This could be an important mechanism through which eosinophils may participate in the downregulation of the inflammatory response. Therefore, I hypothesize that *C1 inhibitor is synthesized and released from eosinophils* and *that eosinophils participate in the downregulation of the inflammatory response through the actions of C1 inhibitor by targeting neutrophil activation in inflammatory diseases.* The presence of C1 inhibitor protein expression in eosinophils stimulated with IFN-γ can be confirmed by subjecting lysates of

highly purified eosinophils to Western blotting. Also, ELISA may be used to measure C1 inhibitor content in eosinophils as well as in neutrophils. Confocal microscopy can be used to show localization and release of C1 inhibitor in eosinophils following incubations with IFN-γ at various time points.

4.6. Conclusion

Eosinophils are enigmatic cells. In asthmatic/allergic inflammation, this cell type has been shown to have a pro-inflammatory role. Yet, the role of eosinophils in innate, nonallergic, antiviral host defense may ascribe a beneficial role for eosinophils. This "double-edged sword" feature of eosinophils is what renders this cell phenotype complex and interesting. The result from the current microarray analysis has opened a new vista to pursue several hypotheses that may provide specific insights into the nature of IFN-γ-eosinophil interactions in health and disease.

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Appendix A. Gene expression to IFN-γ in the human eosinophil

Table A.1. Fold change analysis (≥3 fold) of up-regulated gene expressions in IFN-γ activated eosinophils (4hrs)

Common	Genbank	Fold increase	Common	Genbank	Fold increase
SERPING1	NM_000062	97	NEXN	NM_144573	17
NEXN	AF114264	84	APOL1	AF323540	17
KIAA0241	BC027724	79	ARGBP2	AW138143	17
PLSCR4	NM_020353	49	GBP1	AW014593	17
None	AI017875	42	RPL14	BC000606	16
ADRB1	AI625747	39	NIPSNAP3B	BF510739	16
None	AK023839	39	MICL	AA916861	16
None	NM_014917	37	None	AK024621	15
LAK	AI760166	37	EEF1D	BF690020	15
None	AU157078	34	KIAA0984	AW134976	15
None	AW102716	34	GBP3	AL136680	15
None	AI925518	32	LOC134492	AI291200	15
None	AW872374	30	PDCD1LG1	AI608902	15
C6orf187	BF110556	26	C14orf116	AI279555	15
ANKRD22	AI097229	26	TRY1	AI807285	15
C9orf93	NM_173550	24	GBP5	BG271923	15
TLR8	NM_016610	24	None	BF664545	14
GRIP1	BE672408	24	None	AL535414	14
KCNK1	AL833343	23	FLJ30678	NM_144657	13
FCGR1A	X14355	23	None	AL157448	13
ZNF618	AV704303	23	TNF10	AW474434	13
None	N34548	23	TDRKH	AF227192	13
VMP1	AL541655	21	MGC24665	AW138157	13
OAZIN	AA047234	21	FVT1	BF666293	12
FCGR1A	L03419	21	CXCL9	NM_002416	12
ETV7	AF218365	21	VIL2	AF199015	12
NLGN4X	NM_014893	20	RHBDL6	NM_024599	12
HMG20B	N25429	20	ETV7	AF147782	12
IRTA2	AF343663	20	LY96	NM_015364	11
CML2	NM_016347	18	CASP10	AF111345	11

Table A.1 (continued)

MGC57827	AL135396	11	KCNJ2	BF111326	9
USH3A	AF482697	11	GBP4	BG260886	9
None	BM980001	11	None	BE788984	9
IGSF6	NM_005849	11	GBP1	BC002666	8
BMX	NM_001721	11	GK	NM_000167	8
ENPP2	L35594	11	CASP4	U25804	8
LRFN3	NM_024509	11	SOCS1	AB005043	8
LGP2	NM_024119	11	CXCL11	AF030514	8
NANOS1	AW970089	11	RPS11	BF680255	8
MAT2B	R60683	11	LMTK2	AW206440	7
GBP5	BG545653	11	CDK9	AI040910	7
TNFSF10	U57059	10	GBP2	NM_004120	7
STK3	NM_006281	10	AK3	AI653169	7
VAMP5	NM_006634	10	TNFRSF6	AA164751	6
None	AI830490	10	TNFRSF6	NM_000043	6
FLJ23153	AA650281	10	CXCL11	AF002985	6
T2BP	BG107149	10	GK	X68285	6
None	AF085913	9	GBP1	AW014593	6
TNFSF10	NM_003810	9	CASP10	NM_001230	6
FLJ12571	NM_024926	9	CASP7	NM_001227	6
TLR7	NM_016562	9	PTGER3	D38299	6
KIAA1651	AB051438	9	TNFRSF6	X83493	6
FAM3C	AW241945	9	MHC2TA	NM_000246	6
GBP2	BF509371	9	TNFRSF6	Z70519	6
APOL4	AF305226	9	TNFSF13B	AF134715	6
KIAA1937	AK057963	9	GBP1	NM_002053	5
ZRF1	BC032854	9	FCGR3A	J04162	5
DUSP3	BC002682	9	KCNJ2	AF153820	5
CACNA1D	BE550599	9	IL2RA	K03122	5
DNPEP	AI690583	9	RAB20	NM_017817	5
MGC35048	AK000122	9	TNF13b	AW151360	5
ARHGEF3	NM_019555	9	PDCD1LG1	AF233516	5
ZAK	AI129320	9	IL12A	NM_000882	5
MCL1	AI806486	9	DTNBP1	AF061734	5
MGC20410	AW083820	9	NME7	AI094580	5
None	AI655467	9	BST2	NM_004335	5

Table A.1 (continued)

PLAT	NM_000930	5	LRAP	NM_022350	4
CPD	D85390	5	DHRS9	AF240698	4
APOL6	NM_030641	5	TAGAP	AK025272	4
TLR7	AF245702	5	T2BP	AI147211	4
DTNBP1	AL136637	5	SH3BP5	AL562152	3
NKIRAS1	AI970120	5	RGS1	NM_002922	3
TAP2	AA573502	5	NDST2	NM_003635	3
PSMB4	AA630330	5	IL18RAP	NM_003853	3
TAGAP	AW576600	5	IFI35	BC001356	3
IL2RA	NM_000417	4	RIPK2	AF064824	3
CASP10	AF111344	4	CASP1	M87507	3
STK3	Z25422	4	CASP1	U13698	3
GNBP	AW504458	4	RAB31	BE789881	3
DHRS9	AF240697	4	RAP2A	AI302106	3
LRAP	BE889628	4	JAK2	BC043187	3
BCL6	NM_001706	4	SH3BP5	NM_004844	3
FCGR3A	NM_000570	4	LIMK2	NM_005569	3
GCH1	NM_000161	4	IRF1	NM_002198	3
STAT1	BC002704	4	RHOBTB3	N21138	3
CASP1	U13700	4	PLEK	NM_002664	3
CASP4	AL050391	4	AK3	NM_013410	3
SOD2	W46388	4	TAP2	NM_000544	3
GK	AA292874	4	GPR109B	NM_006018	3
GK	X68285	4	C6orf84	AK023613	3
CARD15	NM_022162	4	GADD45B	NM_015675	3
RHOBTB3	BE620739	4	DDX21	NM_004728	3
IFNRG1	CA447397	4	GADD45B	AF087853	3
GNG10	NM_004125	4	ANXA3	M63310	3
APOBEC3G	NM_021822	4	DUSP5	U16996	3
SKI	AI568728	4	CLECSF2	BC005254	3
PMAIP1	NM_021127	4	RAB31	NM_006868	3
HTLF	NM_002158	4	BCL2L11	AA629050	3
IL15RA	NM_002189	4	TLR10	AF296673	3
CASP1	U13699	4	JAK3	BF512748	3
SOD2	X15132	4	CAPNS2	N32611	3
LAP3	NM_015907	4	CD48	AF143887	3

Table A.1 (continued)

LRAP	BG292389	3	RAB2	AI189609	3
CDKL1	AW976363	3	DNAPTP6	AK002064	3
KIAA0101	NM_014736	3	DAPP1	AA150186	3
RHOBTB3	NM_014899	3	AK3	AK026966	3
CASP1	AI719655	3	APOL6	AW026509	3
DUSP6	BC003143	3	TAGAP	NM_138810	3
STAT3	AA634272	3	ITGA6	NM_000210	3
INDO	M34455	3	PSME2	NM_002818	3
LILRB3	AF009634	3	CPD	AA897514	3
CFLAR	AF041459	3	CCR1	AI421071	3
STAT2	H98105	3	HLA-DOB	NM_002120	3
RGS1	NM_002922	3	MICB	NM_005931	3
ICSBP1	AI073984	3	TOP1	J03250	3
IFNAR2	L41944	3	CD7	AI829961	3
DUSP6	BC003143	3	IFI44	NM_006417	3
PAPSS1	AF033026	3	TLR4	U93091	3
RIPK2	AF027706	3	CCNI	AA020986	3
SMG1	U32581	3	IFIT4	AI075407	3
TLR1	AL050262	3	TLR4	AF177765	3
SP100	U36501	3			
CARD4	NM_006092	3			

Table A.2. Fold change analysis (≤-3 fold) of down-regulated gene expressions in IFN-γ activated eosinophils (4hrs)

Common	Genbank	Fold change	Common	Genbank	Fold change
PTPN22	NM_012411	-37	None	AI620209	-12
SLC19A1	U15939	-34	PEX6	NM_000287	-11
GOSR1	BC012620	-30	MSF	NM_006640	-11
None	AL031718	-30	TBR1	NM_006593	-11
SFRS12	BC017000	-28	PLEKHK1	BF968275	-11
EPS15L1	AV710549	-28	IGVH3	AJ275413	-11
SKP2	NM_005983	-21	FLJ14075	NM_024894	-11
SHREW1	AA835004	-21	KCTD15	W73820	-11
COL9A3	NM_001853	-20	VAMP1	NM_016830	-10
None	AI446234	-18	ARSA	BF111487	-10
PSCD3	AI870144	-18	None	H60543	-10
CASP2	U13022	-18	CD151	NM_004357	-9
JMJD3	AI830331	-17	ATP2A3	AF068220	-9
MONDOA	AF312918	-17	AMPD3	AA919119	-9
ZBTB7	AW027070	-17	SLC7A1	AW452623	-9
SIGLEC7	AJ130712	-17	JUND	AI339541	-9
FLJ14360	BG325646	-17	None	AL359605	-9
None	AL034380	-17	None	BF576710	-9
PAK2	NM_002577	-16	APRT	NM_000485	-9
EHD4	BG540685	-16	YWHAE	U28936	-9
None	BI668074	-15	None	AA877910	-9
ATP6V0A1	NM_005177	-15	None	BF983406	-9
FLJ10154	AU135021	-15	PRKACA	M80335	-9
PCNXL3	AI379451	-15	TRIM8	AI925572	-9
THBS1	AI812030	-14	None	AV709727	-9
CCND2	AI635187	-13	FLJ32731	BF433005	-9
MAZ	NM_002383	-13	None	BE671136	-9
None	N73272	-13	HOXA4	AI473887	-9
IL6R	S72848	-12	None	BF347362	-9
NUP50	AW589982	-12	HYAL3	BC004483	-8
PHOSPHO1	AI016183	-12	HIP14	AF161412	-8

Table A.2 (continued)

KCTD15	AI925361	-8	PLCG2	BC040927	-6
MOBKL2A	BE311936	-8	LTC4S	NM_000897	-6
SQSTM1	AI041019	-8	PDE4A	U18088	-6
MAT2A	NM_005911	-7	ARPC4	AF019888	-6
DDX3X	AF061337	-7	ARHGDIA	AI571798	-6
PXN	D86862	-7	PDLIM7	AW206786	-6
None	J02843cds	-7	SPEC1	AF286592	-6
AGTRAP	AF165187	-7	DGKZ	AI567554	-6
ADAM8	AI814527	-7	DUSP1	AA530892	-5
AKAP1	U34074	-7	LY6E	NM_002346	-5
GSN	BE675337	-7	PTPNS1	D86043	-5
DSCR1	AL049369	-7	ARF6	M57763	-5
PLEC1	Z54367	-7	RGS14	NM_006480	-5
PDE7A	U67932	-7	GPR132	NM_013345	-5
SFXN1	BF593817	-7	REPIN1	BE674760	-5
DOCK5	AL832744	-6	SELO	BC001099	-5
PFN1	NM_005022	-6	PRKAR1A	M18468	-5
TRPV6	NM_004445	-6	GPX1	NM_000581	-5
DSIPI	NM_004089	-6	YWHAH	NM_003405	-5
MGAT3	NM_002409	-6	LGALS1	NM_002305	-5
GPR44; CRTH2	AF118265	-6	SAFB	AI769566	-5
None	AW292635	-6	JUND	AI762296	-5
TREM5	AF427618	-6	RGS14	AF037194	-5
None	BC039329	-6	PIK3CD	U57843	-5
EIF5A	NM_001970	-6	HRBL	AI247494	-5
SMAD3	U76622	-6		AL022328	-5
DEDD	AF064605	-6	CALM1	NM_006888	-5
EWSR1	AF327066	-6	CTSD	NM_001909	-5
PDLIM7	AA086229	-6	THBS1	BF055462	-5
RTN3	NM_006054	-6	THBS1	NM_003246	-5
POGK	AB040946	-6	BTG2	BG339064	-5
None	AI478747	-6	TRAF2	NM_021138	-5
UNC13D	AW189167	-6	UNQ501	NM_004283	-5
C20orf100	AA211909	-6	GPR35	AF089087	-5
UBE3C	BC014029	-6	RIN3	AW027923	-5
LOC117584	BG330374	-6	MKNK2	AA404592	-5

Table A.2 (continued)

IRF2BP2	BG485163	-5	MAP3K2	BG504375	-3
RAB11B	X79780	-5	INPP5A	AF273055	-3
RHOF	BC018208	-4	FCER1G	BC020763	-3
SRPK2	BU155802	-4	ABCC4	BC041560	-3
NDUFB7	NM_004146	-4	COL18A1	BU616656	-3
PTPN18	NM_014369	-4	NDUFS8	NM_002496	-3
CAMK1	NM_003656	-4	RASSF1	NM_007182	-3
IL9R	NM_002186	-4	IL1RL1	NM_003856	-3
PIP5K1A	U78577	-4	IRF2BP1	AI401612	-3
PDE4A	AF073745	-4	TLN1	NM_006289	-3
	BF084105	-4	LSP1	NM_002339	-3
ARRB1	NM_004041	-4	INPP5F	NM_014937	-3
ITM2C	NM_030926	-4	PITPNM1	NM_004910	-3
BCL9L	BG481877	-4	RAPGEF1	NM_005312	-3
TRIB1	AA576947	-4	PTGS1	NM_000962	-3
STYX	BG390493	-4	TCF7	AW027359	-3
PLCD3	NM_133373	-4	PTPN11	L07527	-3
SIGLEC10	NM_033130	-4	SLC6A6	U16120	-3
CASP2	BC002427	-4	SIGLEC8	NM_014442	-3
MAP2K2	AI762811	-4	CANX	AI761759	-3
CCL2	S69738	-4	NFATC1	AW027545	-3
IL5RA	NM_000564	-4	PTPRJ	D37781	-3
DUSP16	AB052156	-4	CDKN2D	U20498	-3
TAPBP	AF314222	-3	TNFRSF10C	AF012536	-3
CALR	NM_004343	-3	IFNGR1	AF056979	-3
ARHGDIA	D13989	-3	CAMK2G	AA284757	-3
PTK2B	NM_004103	-3	ITGB1	NM_033669	-3
PTGER4	AI675173	-3	PITPNC1	BM042439	-3
PTPRCAP	NM_005608	-3	THBS1	AV726673	-3
TNFRSF10C	NM_003841	-3	NMT1	NM_021079	-3
SOCS3	NM_003955	-3	STAT6	NM_003153	-3
GP1BB	NM_000407	-3	VAMP2	BC002737	-3
CSF2RA	NM_006140	-3	MAP2K2	NM_030662	-3
IGSF2	NM_004258	-3	BCL7B	NM_001707	-3
NFKB2	NM_002502	-3	SLA	U44403	-3
IL1RL1	AL117622	-3	CCL4	NM_002984	-3

Table A.2 (continued)

ARHGAP4	NM_001666	-3	PSMF1	BG029917	-3
CDK5R1	AL567411	-3	LAMP1	J03263	-3
MAD	NM_002357	-3	CREBL2	BF438056	-3
PFDN5	NM_002624	-3	SERPINA1	NM_000295	-3
ATP2A3	NM_005173	-3	GSTM4	NM_000850	-3
DGKZ	NM_003646	-3	CHKA	AI991328	-3
MYO9B	NM_004145	-3	CHKA	NM_001277	-3
LAIR1	AF109683	-3	GSTM2	NM_000848	-3
PFDN5	AB055804	-3	CD44	NM_000610	-3
KCNAB2	AF044253	-3	NMT2	AW293531	-3
GALC	D25284	-3	RAB5A	NM_004162	-3
RBM25	BF055107	-3	PSCD3	NM_004227	-3
RHOB	AI263909	-3	PTP4A3	NM_007079	-3
CAMK2G	AI093569	-3	SIAT8D	NM_005668	-3
PRKCI	AI689429	-3	TCF8	NM_030751	-3
OSM	BG437034	-3	ARF1	AA580004	-3
RAB8B	AB038995	-3	TNFSF13	BF448647	-3
CDK9	AI703465	-3	FCGRT	NM_004107	-3
STK17B	AW070323	-3	RAB1B	NM_030981	-3
RGS14	AF037195	-3	SH3BGRL3	NM_031286	-3
PTPNS1L3	AK095499	-3	TMPIT	AF327923	-3
PLD3	NM_012268	-3	NDUFA11	BE741920	-3

Table A.3. Downregulated genes (among the top 100) in eosinophils after IFN-γ stimulation (4 h) grouped according to their known biological functions

Genbank ID	Gene description	Fold change
Genes involved in signal transduction		
NM_012411	protein tyrosine phosphatase, non-receptor type 22	-37
NM_002577	p21 (CDKN1A)-activated kinase 2	-16
S72848	Interleukin 6 receptor	-12
BF968275	rhotekin 2	-11
U28936	tyrosine 3-monooxygenase	-9
M80335	protein kinase, cAMP-dependent, catalytic, alpha	-9
BF576710	protein tyrosine phosphatase type IVA, member 1	-9
AF161412	Huntingtin interacting protein 14	-8
AL049369	Down syndrome critical region gene 1	-7
U67932	phosphodiesterase 7A	-7
AI567554	diacylglycerol kinase, zeta 104kDa	-6
NM_004445	EphB6	-6
BC040927	phospholipase C, γ 2 (phosphatidylinositol-specific)	-6
AI571798	Rho GDP dissociation inhibitor (GDI) alpha	-6
AF286592	small protein effector 1 of Cdc42	-6
Genes involved in metabolism		
AA835004	transmembrane protein SHREW1	-21
AI016183	phosphatase, orphan 1	-12
BF111487	arylsulfatase A	-10
AA919119	adenosine monophosphate deaminase (isoform E)	-9
AW452623	solute carrier family 7, member 1	-9
NM_000485	adenine phosphoribosyltransferase	-9
BC004483	hyaluronoglucosaminidase 3	-8
NM_005911	Methionine adenosyltransferase II, alpha	-7
U18088	phosphodiesterase 4A, cAMP-specific	-6
NM_000897	Leukotriene C4 synthase	-6
Genes involved in cell adhesion		
AJ130712	sialic acid binding Ig-like lectin 7	-17
AI812030	thrombospondin 1	-14

N73272	parvin, beta	-13
NM_004357	CD151 antigen	-9
AF019888	actin related protein 2/3 complex, subunit 4	-6
Cells involved in ion transport		
U15939	solute carrier family 19, member 1	-34
NM_005177	ATPase, H+ transporting	-15
W73820	potassium channel tetramerisation domain containing 15	-11
AF068220	ATPase, Ca++ transporting, ubiquitous	-9
AA877910	ATPase, Ca++ transporting, ubiquitous	-9
BF593817	sideroflexin 1	-7
AW292635	solute carrier family 12, member 3	-6
Cells involved in transcription regulation		
NM_005983	S-phase kinase-associated protein 2 (p45)	-21
AL034380	zinc finger, DHHC domain containing 18	-17
AW027070	HIV-1 inducer of short transcripts binding protein	-17
NM_002383	MYC-associated zinc finger protein	-13
AI635187	cyclin D2	-13
NM_006593	T-box, brain, 1	-11
AI473887	homeo box A4	-9
AI339541	jun D proto-oncogene	-9
AA211909	Chromosome 20 open reading frame 100	-6
NM_004089	delta sleep inducing peptide, immunoreactor	-6
AF064605	death effector domain containing	-6
Genes involved in vesicular transport/golgi		
AL031718	MAPK 8 interacting protein 3	-30
BC012620	golgi SNAP receptor complex member 1	-30
AV710549	epidermal growth factor receptor substrate EPS15R	-28
AI870144	pleckstrin homology, Sec7 and coiled-coil domain 3	-18
NM_016830	Vesicle-associated membrane protein 1	-10
AI041019	Sequestosome 1	-8
AW189167	unc-13 homolog D (C. elegans)	-6
AA086229	Enigma (LIM domain protein)	-6
AW206786	Enigma (LIM domain protein)	-6
Others		
BC017000	Splicing factor, arginine/serine-rich 12	-28
U13022	Caspase 2, apoptosis-related cysteine protease	-18
AW589982	nucleoporin 50kDa	-12

AI620209	dipeptidylpeptidase 7	-12
NM_000287	peroxisomal biogenesis factor 6	-11
AI814527	a disintegrin and metalloproteinase domain 8	-7
BE675337	gelsolin (amyloidosis, Finnish type)	-7
BC014029	ubiquitin-protein isopeptide ligase (E3)	-6
BG330374	Fring	-6
NM_005022	profilin 1	-6
AF327066	Ewing sarcoma breakpoint region 1	-6

Appendix B. Activation response to IFN-γ in the human eosinophil

In sites of inflammation, eosinophils release their granule content to extracellular spaces through a process known as exocytosis, as mentioned in section 1.2.1. A major pattern of mediator release in eosinophils is piecemeal degranulation (PMD), which accounts for 60 - 70% of the degranulation seen in allergic airway tissues [1]. PMD is characterized by the mobilization and secretion of membrane-bound small secretory vesicles following cell activation [1]. Previously, our own group demonstrated that eosinophils undergo PMD following IFN-γ stimulation [2]. This effect was fully inhibited by genistein, a broadly specific tyrosine kinase inhibitor, at 10^{-6}M, suggesting that IFN-γ acts on these cells via its IFN-γ receptor, which in turn activates the JAK-STAT pathway [2]. The STAT pathway leads to activation of nuclear transcription factors, resulting in gene transcription which takes several hours for protein expression [3]. However, it is still unknown whether the JAK/STAT pathway plays a role in IFN-γ-induced PMD in eosinophils. It is proposed that IFN-γ-induced PMD in eosinophils is not dependent on gene transcription events, as PMD takes place within a very short time (15 min) (Figure B.1).

To determine the role of JAK/STAT activation in eosinophil PMD mediated by IFN-γ, flow cytometry technique was used to confirm the presence of the α chain of the IFN-γ receptor on human eosinophils and its increased surface expression in response to IFN-γ (Figure B.2). By western blotting, the presence of JAK-1 and JAK-2 kinases as well as STAT-1 in IFN-γ

stimulated eosinophils were confirmed in both peripheral human eosinophils and the AML14.3D10 eosinophilic cell line (Figure B.3). The phosphorylation of STAT1 protein was measured using anti-phospho-STAT1 antibody (Pharmingen) following 15min of IFN-γ stimulation in AML14.3D10 cells (Figure B.4). The phosphorylation of STAT1 protein was inhibited in a dose-dependent manner by a STAT-1 inhibitor, curcumin (Figure B.4). It was also shown that Rap-1, one of the candidate molecules that may be involved in PMD mediated by IFN-γ in eosinophils (Figure B.1), was expressed in eosinophils both constitutively and following IFN-γ stimulation (Figure B.5).

The major concern in the study of PMD mediated by IFN-γ in eosinophils was the fact that there was no reproducible technique for measuring PMD in eosinophils within a short time frame (10-15 min). Many attempts were made to measure PMD using various techniques listed in the following; Elicell technique (in collaboration with the laboratory of Dr. Peter Weller, Harvard Medical School) [4], marker assays (β-hexominidase assay, eosinophil peroxidase assay) (Figure B.6), confocal microscopy (CLSM) (Figure B.7), flow cytometry (Figure B.8), all of which were unsuccessful. It was concluded that this study could not be further pursued in its current form since it appeared extremely difficult to measure degranulation in a reproducible manner. This problem is not confined to our laboratory.

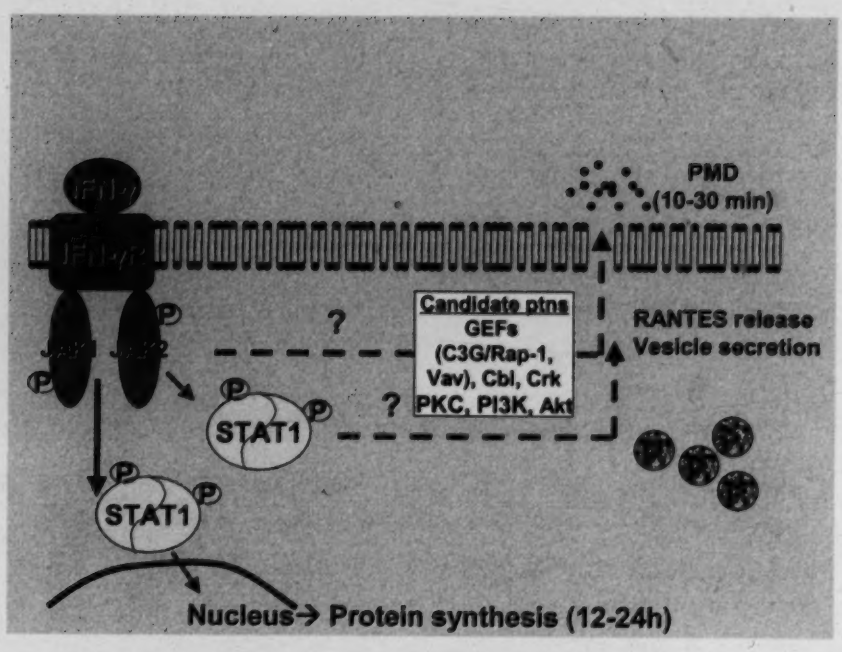


Figure B.1. Model for IFN-γ signaling through a transcription-independent JAK/STAT pathway. IFN-γ has been shown to stimulate rapid mobilization and release of RANTES by PMD in human eosinophils [1]. It is postulated that IFN-γ-induced PMD in eosinophils is not dependent on gene transcription events, as PMD takes place within a very short time (10-30 min). Some of candidate proteins above may have a potential role in this transcription-independent JAK/STAT pathway following IFN-γ stimulation in eosinophils. *Abbreviations:* JAK; Janus kinases, STAT; Signal transducers and activation of transcription, PMD; piecemeal degranulation, GEFs; guanine nucleotide exchange factors, PKC; protein kinase C, PI3K; phosphatidylinositol 3-kinase.

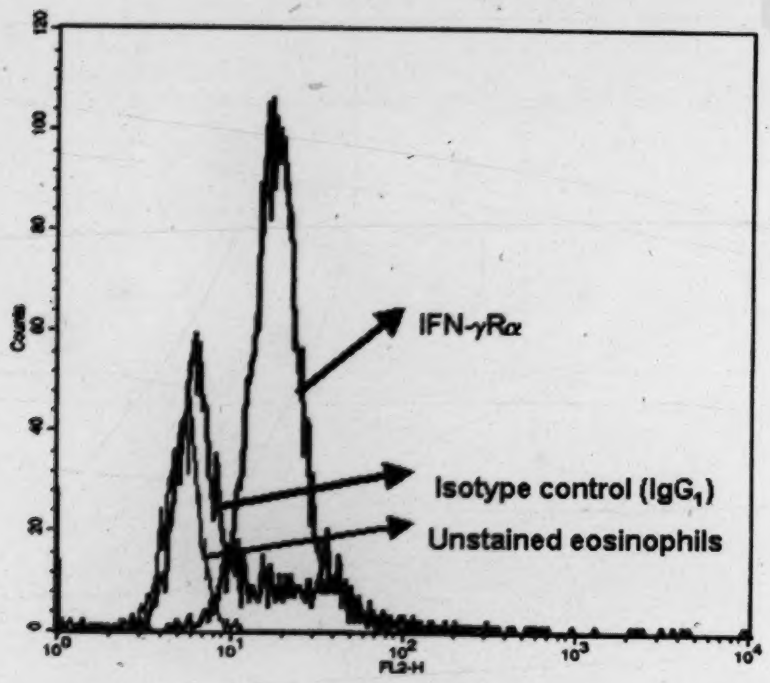


Figure B.2. Flow cytometric analysis of IFN-γR α-chain on peripheral blood eosinophils. Eosinophils were isolated according to Chapter 2. Cells were fixed for 10 min in 5% formalin/PBS before centrifugation at 300g for 5 min. Fixed cells were resuspended in blocking solution containing saponin (0.1% saponin/5% milk/PBS) and left overnight on ice and then labeled with one of the following antibodies (1μg/ml); mouse monoclonal anti-IFNγRα (pharmingen), mouse IgG_1 isotype control (R & D Systems). After detection with secondary antibody conjugated to phycoerythrin, cells were examined by FACS analysis for log fluorescence intensity. Shown is a representative of two similar experiments. The examination of two experiments revealed that eosinophils express IFN-γR α chain.

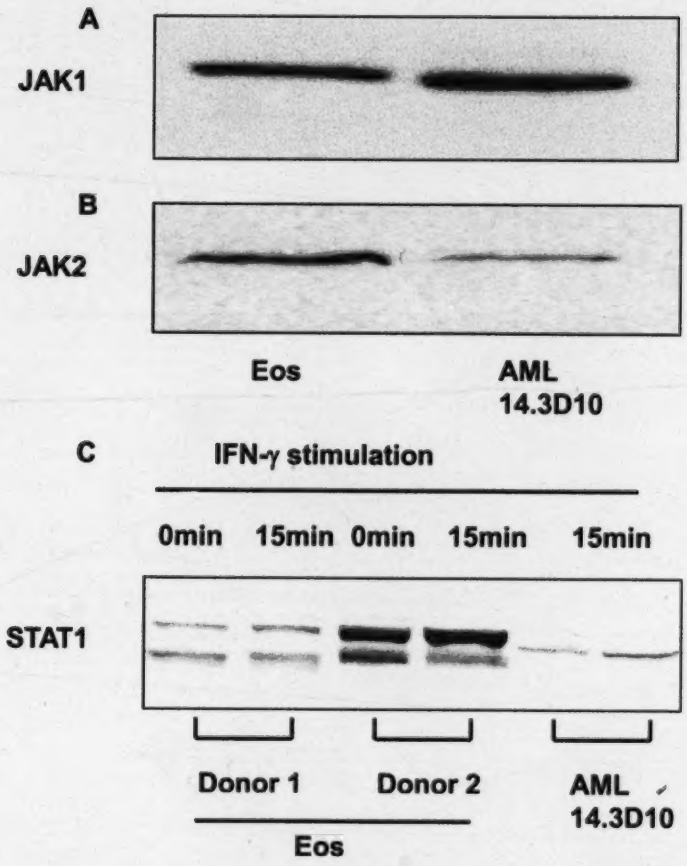


Figure B.3. Expression of JAK/STAT and STAT1 in human eosinophils and AML14.3D10 eosinophilic cell line. Both human eosinophils and the cell line express Jak1, Jak2 and STAT1 constitutively and when stimulated with IFN-γ. Cells were lysed in RIPA buffer after isolation or stimulation with IFN-γ for 15min, and subjected to SDS-PAGE and Western blotting with monoclonal antibodies to A) JAK1 B) JAK2 and C) STAT1 (each at 1:1000) (purchased from R & D Systems).

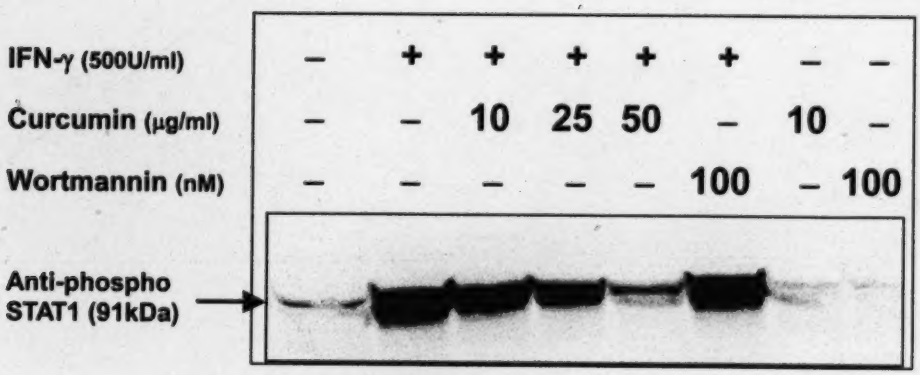


Figure B.4. IFN-γ-dependent tyrosine phosphorylation of STAT1 in AML 14.3D10. The cells were pre-incubated with inhibitors such as curcumin and wortmannin for 15min and 30min, respectively, before incubation with IFN-γ for 15 min. The cells were then lysed and were subjected to SDS-PAGE and Western blotting with anti-phospho-STAT1 antibody. Curcumin inhibited the phosphorylation of STAT1 at increasing concentration. Wortmannin did not block IFN-γ-induced STAT1 signaling in AML14.3D10.

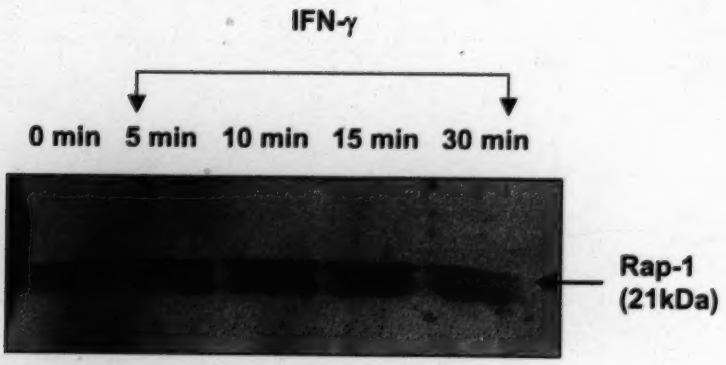
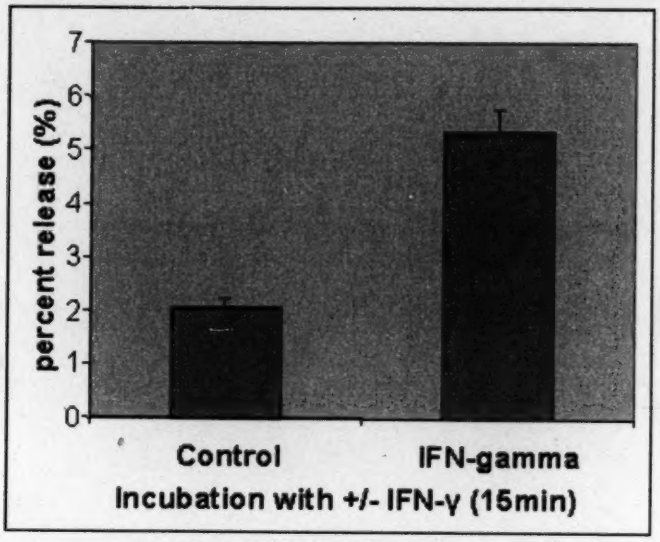


Figure B.5. Constitutive and IFN-γ-induced expression of Rap-1 protein in human peripheral eosinophils. The cells were stimulated with IFN-γ (500U/ml) at 37°C for 0-30 min and were lysed in RIPA buffer. The lysates were then subjected to SDS-PAGE and Western blotting with anti-Rap-1 antibody.

A. Eosinophil peroxidase (EPO) release with IFN-γ stimulation (n=2)



B. β-hexosaminidase (β-hex) release with IFN-γ stimulation (n=2)

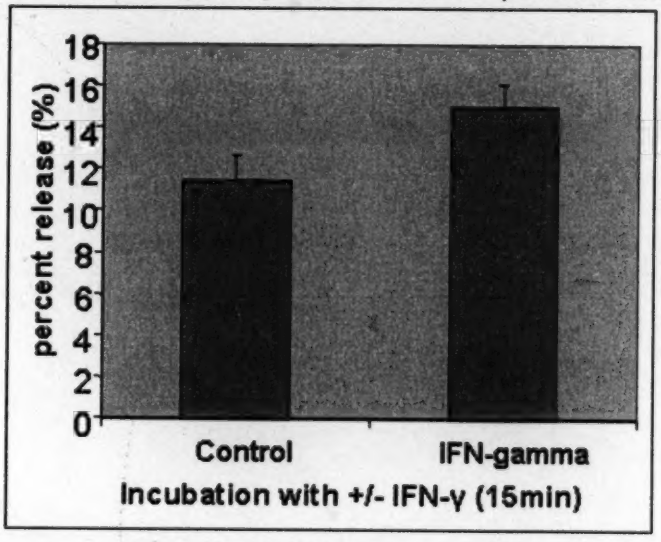


Figure B.6. EPO and β-hex release in response to IFN-γ in human peripheral eosinophils. Human eosinophils were isolated, treated with IFN-γ for 15 min at 37°C, and centrifuged at 300g for 5 min at 4°C. The supernatants were collected and assayed for EPO activity and β-hex activity.

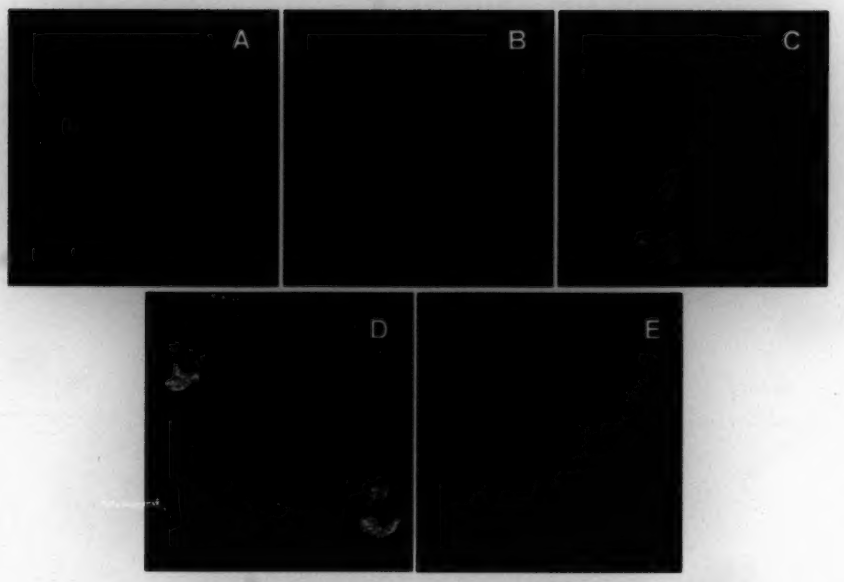


Figure B.7. Confocal laser scanning microscopy (CLSM) of immunofluorescence staining of eosinophils. (A through C) IFN-γ-stimulated eosinophils (500U/ml) (15 min) labeled with BODIPY Green indicating MBP immunoreactivity (A), Rhodamine Red corresponding to RANTES (B), and combined images (C). (D through E) Combined images of RANTES and MBP, depicting unstimulated eosinophils. No significant difference was observed for RANTES immunoreactivity between IFN-γ-stimulated eosinophils and unstimulated eosinophils.

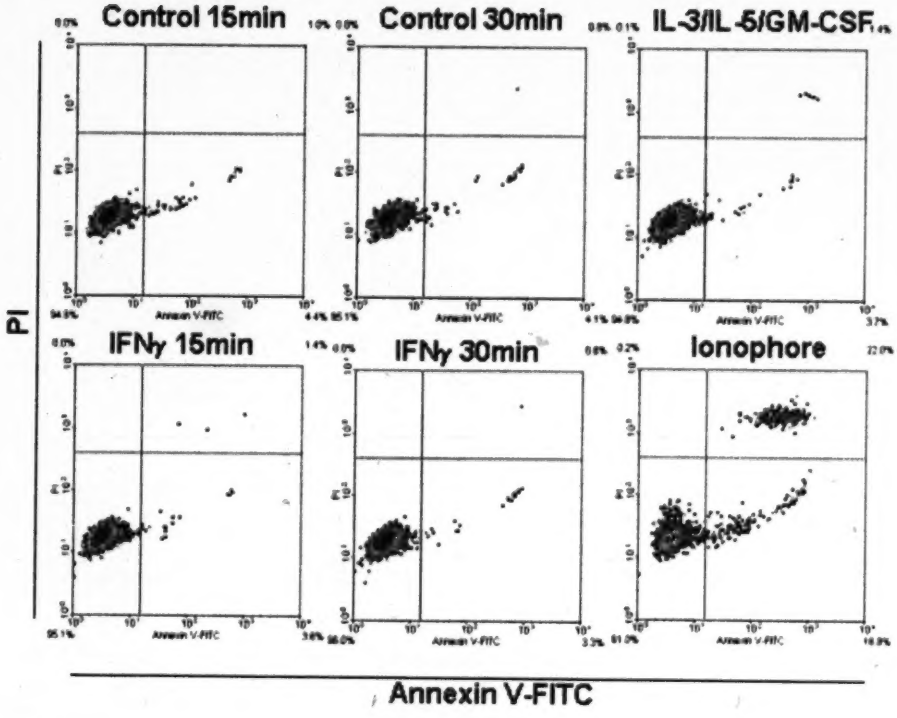


Figure B.8. Annexin V-FITC/PI assay to measure PMD Cells were incubated with no IFN-γ (15 min and 30 min), IFN-γ (15 min and 30 min), IL-3/IL-5/GM-CSF (15 min), and ionophore (15 min). They (10^5 cells) were then suspended in 100 μl of binding buffer (10 mM Hepes/NaOH (pH 7.4) 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated with annexin V-FITC and propidium iodide (PI) following manufacturer's protocol (Invitrogen, ON, Canada). Analysis was performed using FacSCAN flow cytometer (BD Biosciences Mississauga, ON). Thereafter, the cells were analysed by flow cytometry using forward and side scattering to exclude any cell debris and AnnexinV-FITC (530nm) and PI (670nm) specific emission of fluorescence. Cells were categorised as follow: viable: Annexin V^- and PI^-, apoptotic or **degranulation** [5]: **Annexin V^+ and PI^-**, necrotic: Annexin V^+ and PI^+. It was determined that annexin V-FITC/PI assay was not suitable to measure PMD as IFN-γ stimulated cells and the control showed no difference in phenotype (Annexin V^- and PI^-).

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